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DEPARTMENT OF BIOCHEMISTRY
CALCUTTA UNIVERSITY

PROFESSOR BIRESCHANDRA GUHA
(1904—1962)

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FOREWORD

During the inauguration of the Guha Institute of Biochemistry on August 12, 1972, at the Department of Biochemistry, Calcutta University, it was decided to publish a commemoration volume as a mark of respect to late Professor B. C. Guha, the internationally reputed nutritional biochemist, who did much towards the development of Biochemistry and Nutritional Science in India. Not only as a scientist but also as an organizer of science and technology in India, Professor Guha gave ample evidence of his outstanding personality and leadership during his life time.

A number of scientists from India and abroad responded to our appeal and contributed highly interesting scientific articles of the different aspects of modern biochemistry for the commemoration volume. Besides a few research papers presented by eminent nutritionists of India at the all-India Symposium on "Advancing Frontiers of Nutritional Biochemistry" organised on the occasion of the inauguration of the Guha Institute of Biochemistry, are also included in this volume. All these authors are either personal friends or students of late Professor Guha.

We take this opportunity to express our sincere thanks to the teachers, research workers, staff and students of the Department for their untiring efforts and support to make the occasion a success. We are also grateful to some of the firms : M/s. Bengal Immunity Co. Ltd., M/s. B. C. Chatterjee & Bros., M/s. S. K. Biswas & Co., M/s. Satyacharan Ghosh and others for their generous financial help towards meeting the expenses for this great occasion.

We are indeed very much indebted to Prof. D. S. Kothari, the then Chairman, University Grants Commission, who accompanied by his Development officer, Dr. S. K. Dasgupta flew to Calcutta to inaugurate the Guha Institute of Biochemistry under extremely bad weather. Also we like to express our gratitude to Prof. S. N. Sen, Vice-Chancellor, Calcutta University, who in the midst of his heavy responisbilities, kindly consented to preside over the inauguration ceremony. Our thanks are also due to Prof. P. K. Bose, Pro-Vice-Chancellor (Academic Affairs), Calcutta University, for his active interest and advice towards the success of the function. Lastly, we should like to remember gratefully Dr. P. K. Bose, Dr. B. Mukerji, Prof. M. M. Chakrabarti, Mr. S. Kanjilal, Superintendent, Calcutta University Press, for their advice and co-operation.

J. J. GHOSH
D. K. CHAUDHURI
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PROFESSOR BIRES CHANDRA GUHA (1904-1962)

S. C. Roy

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An essential element in the development of a person is his family background, social environment, early education and inspiration. Bireschandra Guha comes of a family, particularly from his maternal side, which to a great extent, symbolized the aims, ideals and resurgent spirit of the contemporary life of Bengal, if not of India. His maternal uncle late Aswinikumar Datta was a staunch nationalist, a patriot of lofty ideals and character and was one of the architects of our national struggle for independence. Born amidst such a family tradition and atmosphere Guha imbibed, as he grew up, many of the characteristics of his illustrious uncle and his time. The efflorescence of cultural life and aspiration for political emancipation which followed in the wake of renaisance of Bengal in the earlier century, also contributed to the early formative period of his life. In fact these influences had an in-built effect in giving direction and depth to the scientific life of Guha in later days.

Guha had a uniformly brilliant academic career as a student of science. He was naturally drawn to Acharya Prafullachandra whose austere and dedicted life to the cause of science also influenced and inspired him greatly. The time when Guha joined the Science College, Calcutta University, may be considered in many respects as a period of efflorescence for the scientific research in India. Raman, Saha, Bose, Ghosh and many others were the illustrious products of the period. They have not only earned permanent names for themselves amongst the scientists of the world, but also extended the frontiers of knowledge considerably. Parallel with this scientific development other branches of knowledge, literature, philosophy, history, etc. were in the height of scholastic achievements. In fact, the University of Calcutta has by that time become a real "centre of excellence and advancement of knowledge."

Young Guha was fortunate enough to join the post-graduate department of the University College of Science in the creative period of the University. To add to that, there were inspiring teachers, like Acharya Prafullachandra, Jagadischandra and a galaxy of outstanding scholars who adorned the various departments of the University. All these factors had also profound impact on Guha for determining the future course of his life.

Krebs rightly says, "distinction breeds distinction or in other words distniction develops if nurtured by distinction." This can be most aptly said

about young Guha. He was practically nurtured by Acharya Prafullachandra, who was in the true sense of the word, his spiritual father. After a brief period of post M.Sc. research in the University College of Science, Guha left for England on a Tata Endowment Fellowship for higher studies and research. He was so very bold in the choice of the subject for his future scientific career. He was an organic chemist by training and discipline. At that time biochemistry was not much known in India except in the field of nutrition and allied areas. Prospect of employment on return to India was highly restrictive if not remote. So it required certain boldness of spirit and self confidence on the part of Guha to take to a path of uncertainty and adventure. He joined the laboratory of Professor J. C. Drummond at the Imperial College of Science and Technology, London, and got his Ph.D. there. He then shifted to Cambridge to work in the laboratory of Professor F. G. Hopkins, which was at that time considered as one of the world's leading centres for advanced study and research in biochemistry. Outstanding achievements of Hopkins and his group had attracted many young brilliant workers there from all over the world. Amongst them were Albert Szent-Györgyi, Hans Kerbs to mention only a few, who were later awarded Nobel Prize and are at present regarded as architects of modern biochemistry. Krebs himself admits that "scientists are not so much born as made by those who teach them research." Guha had the unique advantage of having initial training with Acharya Prafullachandra, the father of modern chemistry in India and later with Hopkins the spiritual father of modern biochemistry. This is perhaps one of the reasons why young Guha shaped up so brilliantly as a scientist in later life. Cambridge was then at the height of scientific achievements and enjoyed the leadership of the scientific world, particularly in atomic physics. Academic atmosphere of Cambridge was quite unique and exciting. Newer ideas and concepts which later revolutionized the whole field of biological sciences were taking some definite shape. These developments in biological sciences and other interdisciplinary fields also shaped the scientific pattern and perspective of Guha as a true scholar and investigator.

Guha had a very liberal training in Cambridge in the Hopkins laboratory. In describing the then atmosphere that prevailed in the laboratory, Kerbs writes, "It was in Hopkin's laboratory where I saw for the first time at close quarters some of the characteristics of what is sometimes referred to as "British ways of life." The Cambridge laboratory included people of many different dispositions, convictions and activities. I saw them argue without quarrelling, quarrel without suspecting, suspect without abusing and criticize without vilifying or ridiculing and praise without flattering."

Love for his country was in the very blood of Guha. The liberal and democratic atmosphere of Cambridge nurtured it into a more concrete and mature form with definite polarization for certain political philosophy. By this time he got his D.Sc. and was now ready to leave for India. Guha's life has been comparatively smooth sailing, marked by uniform success and marred by no untoward events. This sort of successful life has also its compensatory side. Life untouched by any adverse condition or circumstances fails to develop certain toughness of mind and resilience of spirit, which alone immunize a person against disappointment and frustration and make him sturdy enough to pursue his ideals against great setbacks. When Guha returned to India he carried with him a little of Cambridge and its atmosphere of "contagious enthusiasm, broadmindedness and imagination" and above all a deep devotion to science and scientific pursuits. He was full of high hopes, and aspiration. He thought that his scientific achievements and acquisitions will help him to get a suitable place for further development of his capabilities and talents. He however, got a rude shock when he failed to get a professorship in biochemistry in a research institute in Calcutta, simply because of his past political association, which the then alien Government looked upon with considerable askance. He then joined an industrial concern where scope for biochemical research and teaching was practically absent. He soon left that job and joined a college where teaching was at the graduate level without any research facility. All these had a very gnawing effect on his dynamic and superabundant spirit. Because Guha had no hard mental schooling, it was probably very difficulty for him to absorb these disappointments with fortitude and patience. He later got the Professorship of the Department of Applied Chemistry in Calcutta University but his superb training and experience in the field of biochemistry was out of tune with the main objective and teaching programme of the Department. There was practically no laboratory worthy of name for biochemical research in the Department and biochemistry was at that time not even a full subject for postgraduate study in Calcutta University. So Guha was extremely handicapped so far his research was concerned. He lacked requisite fund and a good laboratory for his work. These things, however, do not come without long and hard work. Though Guha might have heard very often from Hopkins that a gold coin can be got in a wayside per chance, but it requires systematic and arduous mining to keep the currency going on. While he was working under such unfavourable conditions, his contemporaris in Cambridge were making history in the field of biochemistry. All these factors in their totality had a profound impact on the inner workings of his sensitive mind. He had so long taken science as his part and mission of life but not as profession of life.

Various setbacks helped to develop slowly an inner dichotomy in him. Guha had considerable mental conflicts before he took to other professions, though in the heart of heart he was for science and scientific pursuits.

For nearly ten years he filled in a number of positions not very much related to biochemistry. There also he showed his outstanding abilities and leadership. In spite of his tremendous success in the different professions and positions, he was the most unhappy man because of his divorce from the real scientific field. The present writer had many intimate occasions to have glimpses of his inner mind during those periods.

Guha was also very fortunate in having for his life's consort an accomplished and illustrious lady like Dr. Phulrenu Guha. In fact she complemented him in every respect.

When his life was full with experience and knowledge garnered from the various walks and ways of life, he perhaps discovered or rather re-discovered, like Goethe's *Faust* that the greatest value in life, that which gives real meaning and the deepest satisfaction is creative achievement. Perhaps with this realization he returned to science fully dedicated to its cause. He now devoted his entire time, energy and his rare leadership and dynamicism in research which soon bore fruits in some spectacular achievements. He was straining too much, as if atoning for his past neglect of science. He was in the height of his research activity, when the end came quite suddenly and unexpectedly. He was, as if, consumed by the very fire which sustained him so long.

Many facets of his myriad mind and multiple positions he held and served with remarkable ability, have been delineated by people who know him more closely and intimately. But to the present writer he is an outstanding scientist, an inspiring teacher and above all a Man and a Soul now redeemed in Self-Dedication.

Let us also not forget, " All our yesterdays have lighted men the way to the scientific creativity of today."

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CONTROL OF THE REDOX STATE OF THE NICOTINAMIDE–ADENINE DINUCLEOTIDE COUPLE IN RAT LIVER CYTOPLASM

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1. A study has been made of the ability of rat liver *in vivo* to maintain equllibrium in the combined glyceraldehyde 3-phosphate dehydrogenase, 3-phosphoglycerate kinase and lactate dehydrogenase reactions, *i.e.* in the system :

$$\frac{[ATP]}{[ADP][HPO_4^{2-}]} = \frac{[pyruvate]}{[lactate]} \cdot \frac{[glyceraldehyde\ 3\text{-}phosphate]}{[3\text{-}phosphoglycerate]} K$$

Attempts were made to upset equilibrium. The [lactate]/[pyruvate] ratio was rapidly changed by injection of ethanol or crotyl alcohol, and the value of [ATP]/[ADP][HPO_4^{2-}] was rapidly changed by injection of ethionine or carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone. 2. The concentrations of the metabolites occurring in the above equation were measured in freeze-clamped liver. 3. Although the injected agents caused large changes in the concentrations of the individual components, near-equilibrium in the system was maintained, as indicated by the fact that the value of [ATP][ADP]/[HPO_4^{2-}], referred to as the phosphorylation state of the adenine nucleotides, measured directly agreed with the value calculated for equilibrium conditions from the above equation. 4. The results are discussed and taken to confirm that the order of magnitude of the value of the redox state of the cytoplasmic NAD couple in rat liver is controlled by the phosphorylation state of the adenine nucleotide system.

The redox state of the NAD couple is linked to the phosphorylation state of the adenine nucleotide system (*i.e.* the ratio [ATP]/[ADP][HPO_4^{2-}]) by the 3-phosphoglycerate kinase and glyoceraldehyde 3-phosphate dehydrogenase reactions (Veech *et al.*, 1970 ; Krebs & Veech, 1970). At equilibrium the following relation holds :

$$\frac{[NAD^+]}{[NADH[} = \frac{[ATP]}{[ADP][HPO_4^{2-}]} \cdot \frac{[3\text{-}phosphoglycerate]}{[glyceraldehyde\ 3\text{-}phosphate]} \frac{1}{K} \qquad (1)$$

where K is the product of the equilibrium constants of the glyceraldehyde 3-phosphate dehydrogenase system and the 3-phosphoglycerate kinase system at pH7.0. By combining eqn. (1) with the equilibrium equation for lactate dehydrogenase a relation is obtained that can be tested experimentally :

$$\frac{[ATP]}{[ADP][HPO_4^{3-}]} = \frac{[pyruvate]}{[lactate]} \frac{[glyceraldehyde\ 3\text{-}phosphate]}{[3\text{-}phosphoglycerate]} K \qquad (2)$$

where K represents the product of the equilibrium constants of the glyceraldehyde 3-phosphate dehydrogenase and the 3-phosphoglycerate kinase reactions divided by the equilibrium constant of the lactate dehydrogenase reaction. The calculation of the phosphorylation state from K and measuremets of the concentrations in freeze-clamped rat liver of lactate, pyruvate, glyceraldehyde 3-phosphate and 3-phosphoglycerate gave values that agreed reasonably well under various dietary conditions with the value obtained from direct determinations of ATP, ADP and P_i. This agreement indicates that the components of lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase systems are near equilibrium *in vivo*, and further that the redox state of the cytoplasmic NAD couple is controlled by the phosphorylation state of the adenine nucleotide system.

The previous work (Veech *et al.*, 1970) was carried out on rat livers from normal, starved and specially dieted rats (high-sucrose, high-glucose and high-fat diets). The present experiments were designed to test whether near-equilibrium in the relation expressed by eqn. (2) is maintained when the phosphorylation state is upset by injection of ethionine or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or when the redox state is upset by the injection of ethanol or crotyl alcohol.

Experimental

Rats

Female rats of the Wistar strain weighing about 200g. were used except in the ethanol experiments, where rats of the Sprague-Dawley strain were used. All were starved for 48 h.

Reagents

Standard analytical grade laboratory reagents were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. All enzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a gift from Dr. P. G. Heytler, E. I. Du Pont de Nemours and Co. (Inc.), Central Research Department, Experimental Station, Wilmington, Del, U.S.A.

Injection of agents

Injection were given intraperitoneally under ether anaesthesia. Details of dose are given in the tables. Rats were killed by dislocation of the neck and the livers were removed within 10s and freeze-clamped (Wollenberger *et al.*, 1960). Further treatment of the tissue was as described by Williamson *et al.* (1967). As some variations in control values were found when experiments were performed at intervals of several months, each series of experiments were compared with control animals analysed at the same time.

Determination of metabolites

Metabolites were determined as follows : lactate and pyruvate by the method of Hohorst *et al.* (1959) ; 3-phosphoglycerate by the method of Czok and Eckert (1963) ; ATP by the method of Lamprecht and Trautschold (1963) ; ADP by the method of Adam (1963) ; dihydroxycetone phosphate by the method of Bücher and Hohorst (1963) ; P_i by the method of Martin and Doty (1949) ; 3-hydroxybutyrate and acetoacetate by the method of Williamson *et al.* (1962). The concentration of glyceraldehyde 3-phosphate was calculated from that of dihydroxyacetone phosphate assuming a [dihydroxyacetone phosphate]/[glyceraldehyde 3-phosphate] ratio of 9.3 (Veech *et al.*, 1969). The concentration of HPO_4^{2-} was taken to be 60% of the total P_i.

Results

Effect of ethionine

Injection of ethionine decreases [ATP] in the liver by the formation of *S*-adenosylethionine at a high rate (Shull, 1962 ; Farber *et al.*, 1964). [ADP] and [PI] also decreased (Table 1). The [lactate]/[pyruvate] ratio increased 2.5-fold in the ethionine-treated animals, but there was no significant change in the [glyceraldehyde 3-phosphate]/[3-phosphoglycerate] ratio.

The calculated and measured [ATP]/[ADP][HPO_4^{2-}] are in reasonably good agreement (Table 1), considering the large changes in [ATP] and [ADP] and in the [ATP]/[ADP] ratio. The latter fell from 1.51 to 0.76 on injection of ethionine. As there was very little change in the [glyceraldehyde 3-phosphate]/[3-phosphoglycerate] ratio it follows that a change in the [lactate]/[pyruvate] ratio compensates for the change in [ATP]/[ADP][HPO_4^{2-}] in eqn. (2).

Effect of ethanol

Ethanol is known to increase the [lactate]/[pyruvate] ratio (see Forsander *et al.*, 1958 ; Smith and Newman, 1959 ; Reboucas and Isselbacher, 1961 ; Field *et al.*, 1963 ; Isselbacher and Greenberger, 1964*a*,*b* ; Freinkel *et al.*, 1965 ; Lieber, 1967). In the experiments recorded in Table 2, this ratio rose from 14.2 to 45.3. This increase was almost entirely due to a rise in [lactate]. The [glyceraldehyde 3-phosphate]/[3-phosphoglycerate] ratio was also increased by ethanol. In this case the increase was caused by a fall in [3-phosphoglycerate].

Although [ATP] was unchanged, [ADP] and [P_i] decreased in the ethanol-treated animals.

In spite of major changes in most of the components in eqn. (2), the agreement between the calculated and measured phosphorylation state is satisfactory and indicates near-equilibrium in this system.

Effect of crotyl alcohol

Crotyl alcohol reacts with liver alcohol dehydrogenase at about the same rate as ethanol, but the crotonaldehyde formed, unlike acetaldehyde, is not a susbtrate of liver aldehyde dehydrogenase (Williamson, 1967).

The change in [lactate]/[pyruvate] ratio was the same with ethanol and crotyl alcohol (Tables 2 and 3) but there were major differences between the effects of

Table 1. *Effect of* L-*ethionine on the metabolite content of rat liver, the redox state of the NAD couple and the phosphorylation state of the adenine nucleotide system.*

The rats were killed and the livers freeze-clamped 6h after intraperitoneal injection of 0.75 mg of L-ethionine/g body wt. The rats were starved for 48h before the injection. Control animals were injected with 0.9% NaCl. Concentrations are expressed in μmol/g fresh wt., as means ± S.D. with the numbers of observations in parentheses. The calculated :

$$\frac{[ATP]}{[ADP][HPO_4^{2-}]} = \frac{[\text{pyruvate}]}{[\text{lactate}]} \cdot \frac{[\text{glyceraldehyde 3-phosphate}]}{[\text{3-phosphoglycerate}]} \cdot K$$

where $K = 53 \times 10^4$ (pH7.0). The (NAD+)/(NADH) ratios are calculated from the means of [lactate] [pyruvate] and [3-hydroxybutyrate]/[acetoacetate] ratios (see Williamson *et al.*, 1967). The P values were calculated on the basis of Student's t test.

Metabolite or metabolite ratio	Control (4)	Ethionine-treated (5)	P
Lactate	0.28 ±0.02	0.85 ±0.45	<0.05
Pyruvate	0.021 ±0.006	0.028 ±0.008	>0.1
[Lactate]/[Pyruvate]	12.6 ±1.72	31.3 ±8.44	<0.005
Glyceraldehyde 3-phosphate	0.0014 ±0.0005	0.0013 ±0.0004	>0.1
3-Phosphoglycerate	0.12 ±0.03	0.18 ±0.05	<0.05
[Glyceraldehyde 3-phosphate]/[3-Phosphoglycerate]	0.011 ±0.003	0.008 ±0.004	>0.1
3-Hydroxybutyrate	1.90 ±0.24	1.46 ±0.41	>0.1
Acetoacetate	0.71 ±0.13	0.47 ±0.11	<0.025
[3-Hydroxybutyrate]/[Acetoacetate]	2.70 ±0.32	3.06 ±0.30	<0.05
ATP	2.15 ±0.11	0.45 ±0.10	<0.001
ADP	1.43 ±0.17	0.59 ±0.06	<0.001
P_i	4.83 ±0.61	3.98 ±0.36	<0.05
[NAD^+]/[NADH] in cytoplasm	715	288	
[NAD^+]/[NADH] in mitochondria	7.15	6.63	
[ATP]/[ADP] [PHO_4^{2-}] measured	533	319	
[ATP]/[ADP] [HPO_4^{2-}] calculated	408	143	

the two alcohols on the [glyceraldehyde 3-phosphate]/[3-phosphoglycerate] ratio and on [Pi] values. The latter rose from 4.39 to 5.92 with crotyl alcohol whereas it fell with ethanol. Ethanol increased the [glyceraldehyde 3-phosphate]/[3-phosphoglycerate] ratio sixfold but there was no significant change in this ratio with crotyl alcohol. In spite of major changes brought about by crotyl alcohol the agreement between the calculated and measured phosphorylation state was good.

Table 2. *Effect of ethanol on the metabolite content of rat liver, the redox state of the NAD couple and the phosphorylation state of the adenine nucleotide system.*

The rats were killed 30 min. after intraperitoneal injection of 2 ml of 1 M-ethanol. The concentrations are expressed in μmol/g fresh wt., as means ± S.E.M. with the numbers of observations in parentheses. The ratios are calculated as described in Table 1.

Metabolite or metabolite ratio	Control (22)	Ethanol-treated (9)
Lactate	0.27 ±0.02	0.77 ±0.08
Pyruvate	0.019 ±0.001	0.017 ±0.001
[Lactate]/[Pyruvate]	14.2	45.3
Glyceraldehyde 3-phosphate	0.0025 ±0.0001	0.0022 ±0.0001
3-Phosphoglycerate	0.14 ±0.02	0.022 ±0.003
[Glyceraldehyde 3-phosphate]/[3-Phosphoglycerate]	0.018	0.101
3-Hydroxybutyrate	1.82 ±0.13	1.85 ±0.17
Acetoacetate	0.65 ±0.04	0.24 ±0.02
[3-Hydroxybutyrate]/[Acetoacetate]	2.8	7.7
ATP	2.32 ±0.08	2.28 ±0.05
ADP	1.48 ±0.03	1.14 ±0.04
P_i	3.69 ±0.12	2.88 ±0.10
$[NAD^+]/[NADH]$ in cytoplasm	634	198
$[NAD^+]/[NADH]$ in mitochondria	7.24	2.63
$[ATP]/[ADP][HPO_4^{2-}]$ measured	709	1189
$[ATP]/[ADP][HPO_4^{2-}]$ calculated	658	1181

Effect of carbonyl cyanide p-trifluoromethoxyphenylhydrazone

This compound, as an uncoupler of oxidative phosphorylation, is expected to decrease the value of the phosphorylation state of the adenine nucleotide system. The effects of the inhibitor on concentrations of metabolites showed considerable quantitative variations but they were always of the same kind. These variations were due to the fact that the effects of the inhibitor were very rapid. Thus [ATP] fell to about one-quarter within 2-3 min. With the dose

given (5 mg.) the animals went into tetanic spasm and died within 5 min. The upset of cellular organization evidently proceeds very rapidly and at the time of freeze-clamping (2-3 min after intraperitoneal injection) the degree of disorganization was liable to differ from experiment to experiment. A representative experiment is shown in Table 4.

Both [lactate] and [pyruvate] increased on injection of the inhibitor and the [lactate]/[pyruvate] ratio increased from 13.2 to 23.5. [3-Phosphoglycerate] increased threefold but [glyceraldehyde 3-phosphate] hardly altered. [ATP] fell fourfold, [ADP] did not change and [Pi] increased twofold, causing a 7.6-fold decrease in the $[ATP]/[ADP][HPO_4^{2-}]$.

Although the changes in some of the components of eqn. (2) are greater than in any other situation tested the calculated and measured phosphorylation states agree remarkably well.

Table 3. *Effect of crotyl alcohol on the metabolite content of rat liver, the redox state of the NAD couple and the phosphorylation state of the adenine nucleotide system.*

The rats were killed and the livers freeze-clamped 15 min. after intraperitoneal injection of 1 ml of 5% crotyl alcohol. Control animals were injected with 0.9% NaCl. Concentrations are expressed in μmol/g fresh wt., as means ± S.D. with numbers of observations in parentheses. The ratios are calculated as described in Table 1. The *P* values were calculated on the basis of Student's *t* test.

Metabolite or metabolite ratio	Control (6)	Crotyl alcohol-treated (5)	*P*
Lactate	0.31 ± 0.05	1.66 ± 0.34	<0.001
Pyruvate	0.026 ± 0.006	0.040 ± 0.006	<0.0025
$\frac{[\text{Lactate}]}{[\text{Pyruvate}]}$	12.2 ± 1.46	41.8 ± 10.1	<0.001
Glyceraldehyde 3-phosphate	0.0022 ± 0.0006	0.0033 ± 0.0006	>0.1
3-Phosphoglycerate	0.18 ± 0.03	0.22 ± 0.10	>0.1
$\frac{[\text{Glyceraldehyde 3-phosphate}]}{[\text{3-Phosphoglycerate}]}$	0.011 ± 0.002	0.018 ± 0.014	>0.1
3-Hydroxybutyrate	1.91 ± 0.24	1.61 ± 0.11	<0.025
Acetoacetate	0.77 ± 0.10	0.53 ± 0.06	<0.0025
$\frac{[\text{3-Hydroxybutyrate}]}{[\text{Acetoacetate}]}$	2.49 ± 0.26	3.04 ± 0.47	<0.025
ATP	2.24 ± 0.22	2.08 ± 0.07	>0.1
ADP	1.51 ± 0.17	1.74 ± 0.22	>0.1
P_i	4.39 ± 0.73	5.92 ± 0.93	<0.05
$[NAD_c^+]/[NADH]$ in cytoplasm	738	215	
$[NAD_m^+]/[NADH]$ in mitochondria	8.14	6.67	
$[ATP]/[ADP][HPO_4^{2-}]$ measured	645	276	
$[ATP]/[ADP][HPO_4^{2-}]$ calculated	488	267	

Mitochondrial redox state

[3-Hydroxybutyrate] and [acetoacetate], indicators of the mitochondrial redox state, were also measured (see Tables 1-4). In three of the four situations when the redox state of the cytoplasmic NAD couple became more reduced, the mitochondrial ratio also changed towards reduction. With ethionine (Table 1) and crotyl alcohol (Table 3) the changes were relatively small (2.7 to 3.06 and 2.49 to 3.04 respectively), but with ethanol (Table 2) there was a 2.8-fold increase in the [3-hydroxybutyrate]/[acetoacetate] ratio (see also Williamson *et al.*, 1969). With carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone there was no change from the control.

Discussion

The experiments indicate that the system represented by eqn. (2) remains at near-equilibrium even when drastic agents suddenly upset either the phosphorylation state (as do carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and ethionine) or the redox state of the NAD couple (as do ethanol and crotyl alcohol). The evidence for the existence of the near-equilibrium is the fact that the direct measurement of the phosphorylation state and the value calculated from K and the concentrations of the intermediates on the right-hand side of eqn. (2) agree reasonably well.

An analysis of the factors that contribute to the maintenance of the equilibrium shows that the change in the [lactate]/[pyruvate] ratio is the same as the change of the factor [glyceraldehyde 3-phosphate][ADP][HPO_4^{2-}]/[3-phosphoglycerate][ATP]. Since the phosphorylation state is not constant under the different experimental conditions, it follows that the ratios [lactate]/[pyruvate] and [glyceraldehyde 3-phosphate]/[3-phosphoglycerate] or [glyceraldehyde 3-phosphate][HPO_4^{2-}]/[3-phosphoglycerate] do not always move in parallel. This is noteworthy because other cytoplasmic redox systems, *e.g.* [glycerol 3-phosphate]/[dihydroxyacetone phosphate] are known to change in parallel with the [lactate]/[pyruvate] ratio because they are at equilibrium with the same cytoplasmic NAD^+ pool (Bücher and Klingenberg, 1958).

It further follows from the agreement between the measured and calculated phosphorylation state that the overall values of ATP, ADP and P_i in the liver correspond closely to those of the cytoplasmic compartment. This is to be expected if the greater part of these metabolites is located in the cytoplasmic compartment and those compartments that readily communicate with the cytoplasm. The latter includes the outer mitochondrial space and probably the nucleus. The concentrations of ATP, ADP and P_i in the matrix are different

from those of the cytoplasm (Klingenberg *et al.*, 1969) but this is not of major significance in the present context because the matrix space constitutes probably no more than 6% of the total liver space. The mitochondria occupy about 20% of the liver space (Lehninger, 1964) and the matrix is only about 30% of the total mitochondrial space (Pfaff *et al.*, 1968).

Table 4. *Effect of carbonyl cyanide p-trifluoromethoxyphenylhydrazone on the content of rat liver metabolites, the redox state of the NAD couple and the phosphorylation state of the adenine nucleotide system*

The rat was killed 2-3 min. after intraperitoneal injection of 5 mg. of carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone. Control animals were injected with 0.9% NaCl. The concentrations are expressed in μmol/g fresh wt. For reasons stated in the text the carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone values are for one experiment. The ratios are calculated as described in Table 1.

Metabolite or metabolite ratio	Control (7)	Carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (1)
Lactate	0.30 ±0.06	3.05
Pyrjvate	0.027 ±0.011	0.13
[Lactate]/[Pyruvate]	13.2 ±7.1	23.5
Glyceraldehyde 3-phospahte	0.0021 ±0.0006	0.0017
3-Phosphoglycerate	0.185 ±0.029	0.58
[Glyceraldehyde 3-phosphate]/[3-Phosphoglycerate]	0.012 ±0.000	0.0029
ATP	2.33 ±0.26	0.58
ADP	1.69 ±0.18	1.58
P_i	4.70 ±0.76	8.96
3-Hydroxybutyrate	1.85 ±0.19	0.69
Acetoacetate	0.76 ±0.12	0.25
[3-Hydroxybutyrate]/[Acetoacetate]	2.48 ±0.32	2.76
$[NAD^+]/[NADH]$ in cytoplasm	816	386
$[NAD^+]/[NADH]$ in mitochondria	8.61	7.35
$[ATP]/[ADP]\,[HPO_4^{2-}]$ measured	489	66
$[ATP]/[ADP]\,[HPO_4^{2-}]$ calculated	549	68

The maintenance of equilibrium in reaction (2) implies that the redox state of the NAD couple depends on the phosphorylation state of the adenine nucleotide system, and vice versa. In view of the reversible interrelation, it may be argued that there is no justification in stating that the redox state is controlled by the phosphorylation state ; with equal justification, it could be said that

the phosphorylation state is controlled by the redox state. However, there are good reasons for assuming that the phosphorylation state is the master factor. The order of magnitude of the phosphorylation state of the cytoplasm is controlled by oxidative phosphorylation and by the translocation of the adenine nucleotides between mitochondria and cytoplasm. Oxidative phosphorylation, because of the obligatory coupling of oxidation and phosphorylation, is so adjusted as to maintain a standard phosphorylation state ($[ATP]/[ADP][HPO_4^{2-}]$) in the cytoplasm of between about 200 and 1000. No explanation has as yet been put forward why in the starved liver the value for the phosphorylation state is lower than in well-fed liver. Factors responsible for variations within the above range may be the rate of ATP consumption and, as the present experiments show, the availability of reducing (or oxidizing) substances in the cell.

Although, then, finer details of the regulation of the cytoplasmic redox state are still unknown, it is reasonable to consider oxidative phosphorylation as the master process controlling the redox state of the NAD couple. The redox state, generally speaking, is therefore a consequence of the phosphorylation state. The reverse sequence of events postulating that changes in the redox state regulate the phosphorylation state is hardly feasible, although the redox state of the NAD couple, by affecting the concentration of the substrates of oxidative phosphorylation (NADH) may, within limits, modify the value of the phosphorylation state.

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A PRIORI APPROACH TO THE SOLUTION OF BIOLOGICAL PROBLEMS

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During the past several years our laboratory has concentrated on two main problems—the molecular principles of membrane construction and the mechanism of energy transduction in biological systems. Our efforts on each of these two problems have followed a similar course. First we embarked on a search for a principle and then zeroed in on a principle sufficiently versatile to rationalize the existing body of experimental information—the bimodal principle for membrane construction (1-3) and the electromechanochemical principle for energy transduction (4, 5). As we have reflected on the approach which was taken in arriving at the respective principles and in testing the accommodation of the experimental data to these principles, we recognized that we had introduced a new dimension in the tactics of problem solving. The present article is addressed to this new dimension which we have elected to refer to as the *a priori* approach to the solution of biological problems.

I. THEORY OF THE *a priori* METHOD

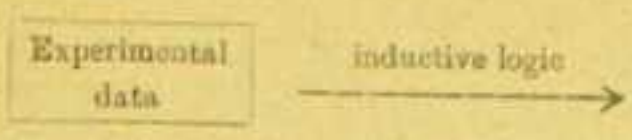
There are two approaches to the solution of scientific problems—the classical or inductive method and the *a priori* or deductive method. Scientific problems may be divided into two classes ; those which can be solved by the inductive method and those which cannot at any given period. The latter problems can only be solved by the *a priori* method. The inductive method involves extracting the correct conclusion from a set of experimental observations based on established physical principles and rigorous logical reasoning. Of primary importance in the application of the inductive approach are the appropriateness of the physical principles invoked and the soundness of the logical processes. Given these two conditions, the correct conclusions follow automatically from experimental facts just as input data can be transformed into output information in a predictable manner by an electronic computer. We will refer to scientific

problems which are amenable to inductive solution as "simple" problems and to those which can be solved via the *a priori* method as "complex" problems. The solution to a complex problem by our definition lies beyond the capability of the inductive method. The question of what constitutes the distinguishing features of simple and complex problems as defined here is an important problem from the point of view of the philosophy of science. Simple and complex problems may be distinguished on the basis of at least two criteria : (1) the number of variables actually implicated in a problem as compared to the number of variables experimentally measurable and (2) whether the solution to a problem presupposes the validity of the existing theoretical framework or is sought outside of the paradigm (7). In certain cases these two criteria may not be mutually exclusive but overlap each other. In terms of the first criterion, simple problems may be regarded as those problems for which there are sufficient experimental and/or theoretical data for arriving at a unique solution based on inductive logic, while complex problems may represent those problems for which there are incomplete experimental and/or theoretical data so that inductive logic leads to multiple solutions. In the light of the second criterion, simple problems may be thought of as those problems whose solutions are designed either to confirm or at best to refine the existing paradigm, whereas complex problems involve solutions that are no longer predictable nor formulatable within the paradigm and thus lead to the establishment of a new theoretical framework.

The *a priori* method encompases two distinct phases—the initial phase when a solution to a complex problem is conceived of in terms of a model deduced from available experimental information plus intuition (the" pre-model phase ") and the final phase when the model is tested in terms of its ability to fit theory to facts, to organize a wide variety of experimental data into a set of interrelated categories and to predict new experimental results (the "post-model phase"). Whereas the solution to a simple problem is validated by the rigor of the inductive logic and the soundness of the physical principles invoked, the solution to a complex problem cannot be validated unambiguously in a similar manner, and hence requires a whole new tactic. The proof of the solution to a complex problem involves showing the ability of the model to "fit," to "organize" and to "predict". As mentioned above, these three capabilities are the deductive aspects of the model. The *a priori* method consists of both the inductive component in the pre-model phase and the deductive component in the post-model phase. In addition, the *a priori* approach involves a third component which may be referred to as the "creative act" or "intuitive jump" which bridges the gap between the available experimental information and the final

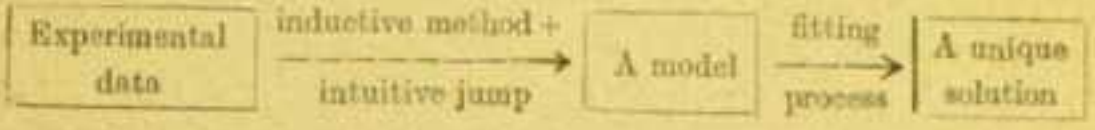
solution. The characteristic features of the inductive and the *a priori* methods are schematically shown in the following equations :

INDUCTIVE METHOD



.....(1)

A PRIORI METHOD



.....(2)

It is to be noted that the inductive method starts with experimental data and ends up with a solution whereas the *a priori* method begins with experimental data, progresses to a model and then returns to the experimental data in the fitting process. The deductive aspect of the *a priori* method is necessary because the pre-model phase involves an extra-logical "intuitive jump".

There are two different sets of ground rules applicable to the two methods of scientific research. In the application of the inductive method the exercise of one's intuitive capacity is taboo. And yet this very faculty plays a critical role in the practice of the *a priori* method as shown in Equation (2). In the evaluation of the model achieved via the *a priori* method, therefore, the important question to ask is not *how* one arrived at the model but *how well* the model fits theory to facts. This question of fitting assumes a fundamental significance in the *a priori* method, whereas the fitting process is not as critical in the inductive method.

If one accepts the view that all scientific problems can be divided into simple and complex problems and that the *a priori* method is mandatory for the successful solution of complex problems, one is forced to make a tactical decision before embarking on any scientific inquiry. Too often this tactical question has been ignored, and many investigators have blindly followed the classical inductive approach in their attempts to solve complex problems with frustrating results. It is our contention that all of the modern biological problems of major importance such as mitochondrial energy transduction, structure of biological membranes, photosynthesis, active transport, mechanism of carcinogenesis, etc. belong to the category of complex problems as defined above and consequently can be solved at this time only via the *a priori* method and not

by the classical inductive approach. This also means that any proposed solution to a complex problem arrived at deductively via the *a priori* method must be evaluated in the light of a new standard distinct from the standard applicable to the solution of simple problems. Scientists trained in what T. S. Kuhn (7) refers to as normal science are well versed in the standard by which solutions to simple problems are to be judged. Their training, however, does not provide any frame of reference when it comes to evaluating solutions to complex problems. If they were to apply the paradigm standard to complex problem solving they would most likely experience disillusionment and confusion. Such undesirable consequences can be avoided only if investigators are aware of the differences in the ground rules to be observed in solving simple versus complex problems. One important difference between the two standards is that solutions to simple problems are formulatable in rigorous details but solutions to complex problems are not always so expressible. In the latter case, it is often necessary to employ detailed descriptions merely as heuristic devices as long as they are instrumental in conveying the central concept underlying the solution of a complex problem.

In applying the *a priori* method to complex problems such as the molecular principles of membrane construction, it became necessary at the very start to come to grips with the question of the universality of biological principles. Are all membranes constructed on the same principle or is each membrane a law to itself? Our view has been unambiguously that the principles which underlie the fundamental biological phenomena (the genetic code, the coenzymes, energy transduction, citric cycle, ATP as an energy donor, etc.) are universal (7). Given this assumption then the test for the correct constructional principle of membranes would have to be its applicability to every known kind of membrane in essential features. While the criterion of universality may appear to place an insuperable burden on any proposed principle, in practice this criterion is enormously useful since the wide variety of membrane systems found in nature makes it easy to check and double check the fit of the model to the experimental facts. Moreover, the requirement of a general solution for all membranes helps to emancipate the investigator from the shackles of too limited a frame of reference.

In ferreting out a principle such as the bimodal principle of membrane construction, there are unexpected emergent developments flowing from this principle. For example, the bimodal principle (that both proteins and phospholipids nest together in paired arrays within the membrane continuum) led to the following major developments : (1) there is a special kind of protein in the membrane continuum which has a major sector of its surface predomi-

nantly nonpolar (1-3); (2) the intrinsic proteins of membranes are bimodal whereas the extrinsic proteins (the proteins associated with or attached to but not intrinsic to the continuum) are not (8); (3) proteins and phospholipids are arranged in paired arrays within the membrane continuum (1-3); (4) the pairing of proteins and phospholipids leads to separate domains of protein and lipid and to potential crystalline properties of biological membranes (3); and 5) the double tier character of membranes provides an ideal arrangement for generation of an electric potential by charge separation (4). It was not until the bimodality of membrane proteins was recognized that these simple but fundamental properties of membrane systems were recognized. Surprisingly enough, the experimental foundations for each of these five developments has been laid for some years but until the fog of imperfect and constricting theory was dispersed, the true meaning and implication of the facts could not be recognized.

II. Generalists and the *a priori* method

The practioner of the *a priori* method has to be a specialists in fitting theory to experiment and this role calls for a special kind of training and prepatation. The professional theoretician and the highly specialized experimentalist represent the two extremes of the spectrum of skills which the practioner must avoid. The middle-man role of fitting theory to experiment requires the capability to search out and comprehend the wide range of physical theories that have to be considered in the selection process. But the development of physical theories is an activity apart from the application of these theories. What we are suggesting is that the fitting process requires a generalist approach and that the style required for the rigorous development of physical theories is hardly conductive to the growth of a generalist. Much the same kind of considerations apply to the experimental realm. The practioner of the *a priori* method must be a connoisseur of experiment and the experimental method. He cannot afford the luxury of concentrating on any one experimental approach. He must be aware of the totality of experimental findings if the fitting process is to be successful. Coming up through the ranks of the highly specialized experimentalists may hardly be the ideal preparation for the fitting role of the practitioner of the *a priori* method. The myopia and the limited horizons which are inevitable consequences of extreme specialization in the experimental sciences can be detrimental to the proper development of a generalist. As we see it the art is to be at home in both theory and experimental science as an amateur and not as a professional. Excessive knowledge in any one field whether theoretical or experimental can inhibit the development of the highly flexible attitudes which are required for the fitting of theory and experiment.

III. Problem Solving and the Tactics of Research

There is a fallacy commonly accepted not only by laymen but by many scientists and administrators of research funds that given enough investigators and support, all major problems of biology (*i.e.*, complex problems) can be rapidly solved. In fact a strong case can be made for the opposite thesis, namely that the greater the number of investigators concentrating on a given complex problem, the less is the likelihood of a solution within a reasonable period. The explanation for this paradox is that when a large number of investigators concentrate on the same problem, cliches of theory inevitably become canonized and the activation energy required to break out of these cliches becomes so large that progress soon grinds to a halt. The theory of membrane structure provides a good example of just this paradox. The number of investigators who have concentrated on the problem of membrane structure is legion. Since early 1930 the Davson-Danielli model (9) had become the accepted model of membrane structure and this domination of biological thought by the model was complete for some 30 years. Although evidence has been mounting for the past decade that the model could not accommodate the hydrophobic nature of lipid-protein binding or the hydrophobic character of intrinsic membrane proteins, such was the power of entrenched dogma that until very recently dissenting voices found no forum either in the established journals or in national and international conferences. That this type of rigidification is common in science is of course well known (7). The point at issue is not to emphasize a traditional weakness of the scientific establishment but rather to suggest a remedy. In the experimental sciences, the emphasis is on experimentation and theory is considered a necessary evil. To minimize this evil, *ad hoc* and often unsound theory becomes elevated to indisputable dogma and from then on in the doors to further discussion of theory are closed. Thus the stage is set for the emasculation of the field.

If investigators were concerned with problem solving and with the tactics of problem solving, it would be impossible for an untenable theory to dominate a field for any considerable period. The problem solvers would immediately have tested the fit of theory and experiemnt and found the theory to be wanting. The search for a more acceptable theory which fitted the facts would then be initiated forthwith. That this fitting process is not always an integral part of contemporary biological science is a serious indictment. That critical testing of the accepted model of a membrane should have been postponed for 30 years provides strong support for the notion that balance in scientific styles is a pre-requisite for rapid progress. Were there practioners of the *a priori* method in

the membrane field, the weaknesses of the unit membrane model would have been recognized long before the late sixties and the search for a better model would have been initiated at least ten years earlier than has been the case. Problem solving is more than diligent and rigorous experimentation. The tactical component in research is crucial. It is for this very reason that the *a priori* method of problem solving can play a vital role in the scientific effort. For it is only this method that is capable of pointing up the faulty cliches of contemporary thinking and of focussing on the fit of experiment and theory. Experimentalists often show the most tolerant attitude to theory which has become superannuated by the facts.

Perhaps it would be appropriate to consider the problem solving capability respectively of the classical inductive method and the *a priori* method. This directional quality is relatively low in the former and high in the latter. One can experiment widely and effectively without thinking about solving prolems but it is virtually impossible to practice the *a priori* method without thinking about or planning for solutions. In that sense the *a priori* method has a directional arrow often lacking in the classical inductive method.

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ACADEMIC ASPECTS OF APPLIED RESEARCH ON FOOD PROTEINS

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In every country, regardless of its state of development, biological research should, among other things, be useful. Each scientist engaged in it should be ready to explain comprehensibly and convincingly how what he is doing may be expected to improve the nutrition, health or comfort of the community. Quesnay, who found the "Physiocratic School" in the 18th century, maintained that the whole of society was parasitic on farmers: that may be an extreme position. But it is undeniable that food is a more fundamental requirement than clothing or housing, and peoples' food needs are much more uniform than their real or imagined medical needs. It would be reasonable, therefore, to give precedence to the study of food production in the hope that, by the end of this century, the supply will be adequate throughout the world.

Although use should be its primary purpose, research will not be successful if it is planned too narrowly. Both for the intellectual satisfaction of the scientist, and for the logical development of the subject, some exploration in depth is reasonable. Furthermore, the work need not necessarily be planned so as to yield practically applicable results immediately—work is useful even although applications may not flow from it for 10 years. The appropriate division of effort between work for which an immediate application can be seen, work with longer-range application, and work undertaken to round-off the subject, will depend on the resources that each community is prepared to devote to research.

In technically developed countries, the amount of money, per head, spent on applied research is greater than in the developing countries. But, because of greater affluence, so much impractical research is also done than it tends to dominate the research scene. Most of this impractical research shows little originality and may not even present much evidence for the scientific competence of those involved in it. One gets the impression that a certain species, or enzyme is being studied for no better reason than that no one has higherto taken the trouble to study it. This is what fills—one might say clutters—the literature. It is unfortunately often taken as a model of what scientific research should be. Research does not become original merely because it is concerned with something hitherto uninvestigated, if the objective, methods and concepts are

all conventional. There is, admittedly, a random element. Something novel may emerge and be noticed. This is just as likely to happen in thoroughly practical research ; especially in a new environment. A country without extensive research endowment should be wary of following the bad example set elsewhere. Only the wealthy can afford to run a scientific band-waggon. A real difficulty arises over who is to judge, and the only satisfactory solution is self-criticism. How practical this solution is, is debatable. No one would wish to discourage an academic research worker who has a genuinely original concept or method ; however, those who supply the money are entitled to call for more evidence for genuine originality than is usually called for in affluent countries. Judging from the incidence of genuine originality in affluent countries, it would not be reasonable to expect more than 2 or 3 for each million of the population.

Experience suggests that there is a correlation between the originality and initiative of scientists and their ability to get interesting and novel results with simple equipment. In all countries, most of the grumbles about the absence of expensive equipment come from the less imaginative : they may be safeguarding their self-esteem by blaming the facilities for their own lack of productivity. Illogical administrators have sometimes suggested that originality could be stimulated by worsening the conditions for reaserch. That is obviously absurd. The fact remains, however, that successful research depends on the people who are doing it rather than on the equipment with which it is done. Without certain types of equipment, certain types of research cannot be undertaken. But there are other types of research. It is woth bearing in mind that the research on which the present agricultural prosperity of Western Europe and USA depends was done with facilities and equipment that now seen primitive. It is also worth bearing in mind that many of the early attempts to set up self-sustained European settlements in Australia and USA, countries now embarrassed by food surpluses, failed because of starvation. There is, therefore, no reason to think that countries that have got into the habit of importing much of their food, will not become self-sufficient when adequate applied research is done. This research is often approached unreasonably timidly. The productive attitude is : " this problem will be solved by someone sometime, it might as well be solved by me now."

The early emigrants from Europe, contending with the problems of the countries in which they were trying to establish settlements, had one immense advantage over those who are now trying to improve nutritional and other standards in somewhat similar countries—they were compelled to be self-reliant because there was no one else to whom they could turn for help. Had there

been International Agencies and "experts," these early emigrants would no doubt have striven less and asked for help more. They were no more intelligent or hard-working than people today and their moral and ethical outlooks were often deplorable. Circumstances forced them to develop the necessary new techniques of farming.

Reliance on "experts" has two disadvantages : it damps local initiative and "experts" are seldom expert enough. An expert can usefully be called in to do a standard job that is precisely the same as one already done elsewhere —that amounts, in effect, to installing a piece of equipment. The equipment may not be suitable for the job intended, but the process of installation will probably be impeccable. The real problems of agriculture are seldom of this type because the conditions in one region are seldom identical with those in which the "expert" gained expertise. Visitors to developing countries are told many stories of "experts" who, after a brief inspection, have advised the use of certain crops or methods and then have gone away without waiting to see how mistaken the advice was. These stories are often told as jokes at the expense of scientists. This is a pity because the people concerned were not being scientific. The scientific approach would be for the adviser to say "I have seen land and climatic conditions somewhat like this where such-and-such a procedure was successful. Let us try it on a small scale, see what happens, try it next year on a larger scale, and so on." Two pionts are important. Unless the adviser is familiar with similar conditions, the advice given could, more economically, have been culled from books. The adviser should look on what he is doing as an experiment and remain in contact with it until a conclusion has been reached. Otherwise, the same mistaken advice may be given repeatedly.

Academic scientists, especially when they lack prolonged personal experience of the evironment in which their advice might be followed, should therefore hesitate before offering advice, even in the cautious style "these are things that should be tested," rather than "these are things that should be done". My selection of food proteins as an area of practical importance is not meant to suggest that the protein component of a diet is more important than energy, vitamins or minerals. All are essential. But it will probably be more difficult to ensure world-wide adequacy of protein than of these other dietary components.

In inland areas, sharply contrasted problems arise when two main protein sources are considered. The annual yield of meat from ruminants grazing and browsing on savannah is often unsatisfactory. Possible reasons for this are : inadequate supplies of major plant nutrients and trace elements in severely leached soil, unsuitable types of vegetation, and the use of species or breeds

of animal that are not ideal. Work on the animals, possibly involving the domestication of novel species of ruminant, and of non-ruminants such as the capybara and kangaroo, will be expensive and will need elaborate organisation. Work on nutrients and plants is simpler and could start at a University, using pots holding as little as 500 g of savannah soil each, and then be extended to plots at some convenient site in the savannah. This is how the right treatments for unproductive soils in Britain, USA and elsewhere were found ; enough has by now been published for the work to begin immediately. A point that must be recognised at the start is that no soil can remain productive for long if its product is removed for consumption elsewhere. Soils differ in the length of time for which they will tolerate " mining " or " quarrying, " but ultimately the elements that are being removed must be put back. The defect of many leached inland tropical soils is that their supply of some of the nutrients is already exhausted so that a state of affairs that would arise only after several years of exploitation in more favoured sites, exists at the very beginning.

This work will involve testing hundreds, or even thousands, of different plant and fertiliser combinations, and many scientists regard such work as unglamorous and without the up-to-date tone of studies on the minutiae of the behaviour of RNA, DNA etc. which have now become the routine in laboratories in Europe and USA and fill the international journals. It should be borne in mind that it was just such practical work that established the well-deserved reputations (*e.g.* Fellowship of the Royal Society) of the scientists who cleared up the problems of cobalt deficiency. As more bio-elements are discovered, it naturally becomes more difficult to find new ones. But the roster is probably not yet complete and an ancient leached soil is a likely place to look for those that remain.

Tropical rain forest is another immense underexploited region. When cleared and farmed in a conventional manner, the unprotected soil tends to erode. Forest trees can be replaced by rubber, cocoa etc. but the product does not supply food to the inhabitants except through the somewhat uncertain cycle of sale overseas ; to get foreign currency ; to finance food imports. Imported food, not unexpectedly, tends to be eaten in towns ; it is already becoming clear (FAO, 1969 ; Hidalgo *et al.* 1970 ; Mata, 1971) that people are often better fed in towns than in the country. The search for protein-rich crops that will flourish in regions where it rains nearly every day, so that seed-crops have difficulty ripening, has been superficial. The edible underground parts of yams and similar crops tend to contain a smaller percentage of protein than cereal or legume seeds. Their nitrogen content can be as great as 2.5%, but there is no evidence that most of that occurs as protein rather than as useless

(or even harmful) components. The point merits investigation. A protein-rich yam would be very useful ; but it would not completely solve the problem of erosion because it would not give complete ground-cover throughout the year.

In many parts of the world, cover-crops are used to prevent erosion—but they are then wholly or to a great extent, wasted. Research directed at finding a protein-rich food crop that maintains continuous ground-cover is urgently needed. Ideally, it would be a perennial, part of which can be eaten without more processing than can be managed in the kitchen. The yield from such a crop would probably be small. A crop from which leaf protein can be extracted would given a greater yield though of less familiar material. When Guha was working in Cambridge, we often met because we shared an unwillingness to be constrained by conventional working hours. Conversation ranged widely : from immediate political matters, through the social value of scientific research, to more esoteric themes such as the vitamin research on which he was engaged, and the work on plant viruses that I was starting. The need for new sources of protein was one of our themes. When the war caused a slight food shortage in Britain, and intensified the food shortage in India, we both began extracting leaf protein on a technological scale. Other responsibilities diverted Guha from this work, but I have continued it.

At Rothamsted we can get 2 tons of extracted dry protein from a hectare in a year (Arkcoll, 1971), the yield is 3 tons in Mysore ; it should be possible to get 5 tons in a region wit regular rainfall and no period of cold weather. The process of extraction and purification is simple enough to be used in a large village, and the product has better nutritional value than any seed protein (references to papers dealing with leaf protein are in Pirie, 1969 ; 1971). Careful agronomic work is now needed to design systems of husbandry that will give optimum yields ; this has started in Aurangabad, Calcutta, Coimbatore and elsewhere. It is reasonable to expect many intriguing new phenomena in plant physiology to emerge when serious effort is put into attempts to maximise vegetative growth.

The possibility of using leaf protein as a human food broadens the basis of crop selection. Work on maximising photosynthesis and nitrate reduction was hitherto not practically useful unless done on a species with leaves palatable to people or stock, or on a species that was efficient at translocating the protein into seeds or tubers. Even when attention is confined to the leaves, where the initial synthesis takes place, choice of plants is not unrestricted ; the protein must be readily extractable by the methods now used or envisaged. Thus, sunflower is an outstanding synthesiser of leaf protein, but when the leaves

are large enough to give a useful harvest, the protein extracts poorly. Poor extraction can arise in different species from many different causes. Sometimes the trouble is physical and could be overcome by more vigorous pulping or by digestion with cellulase—a still underexploited enzyme (Pirie, 1953). Acid leaves, such as sisal, might be satisfactory if pulped in the presence of alkali. Extraction from the mature leaves of some plant varieties and species is impeded by polyphenols and other tanning agents, the difficulty may be circumvented by using younger leaves or other varieties. Work on these factors is not of interest in connection with leaf protein extraction only, for they are also responsible for the rejection of many species as forages or vegetables. Plants are now often dismissed as "unpalatable" or "not suitable for protein extraction" without any effort being made to identify the defect. A thorough, academic, study of the defects in some at-present unuseable species is an essential prelude to agronomic work on optimal conditions for their husbandry.

The essence of these suggestions is that the skilled academic work that is now done on plant breeding, physiology and biochemistry would become more useful, and be no less interesting scientifically, if it were prompted by a recognition of the protein shortage and a realisation that the better use of plants will contribute to removing it. The lines of work suggested would fit easily into the usual departmental divisions of a University, and a reasonably energetic student should be able to get definite results within two or three years. Some other potentially useful lines of work would not be so easy to accommodate.

Several publications suggest that a plot carrying a mixture of plant species or varieties can out-yield a plot with only one. The point should be cleared up because it is of great theoretical, and possibly practical, importance. Detrimental exudates and effluvia are well known : are there others that promote growth in natural conditions ? Hitherto, work on the subject has been inadequately replicated and controlled : any research now undertaken should be impeccable.

Tropical waterways are constantly threatened, or invaded, by water weeds. It is widely assumed that these weeds are useless and that cutting or the use of herbicides are the only satisfactory means of keeping canals and irrigation ditches clear. These assumptions may not be valid. I have seen water hyacinth being collected as cattle and pig food, and I have seen these animals browsing readily on the weed in unfenced ponds. There is no evidence that it is beneficial. It would be easy to get this evidence by gradually replacing part of a conventional diet for pigs or ruminants with hyacinth, and studying the effect on performance. It may be that the hyacinth would need pre-treatment, *e.g.* chopping, crushing or heating. This work could, therefore, conveniently form

part of any programme of leaf protein extraction. Guha extracted protein from hyacinth and it has been extracted by others recently. The important point is to test the assumption that the only thing to do with a weed is to try to eliminate it. As with mixed cropping there are several inconclusive papers stating that hyacinth and other weeds are good feeds, and that manatees will thrive on them and, at the same time, keep canals clear. These points still need to be established unequivocally.

These suggestions are illustrative rather than comprehensive. They reflect my personal interests, and those with different interests would have produced an equally relevant and academically interesting set dealing with other sources of protein. For example Jaffe (Venezuelan National Program in the International Biological Program, 1968) is examining 400 varieties of *Phaseolus vulgaris* to see whether any of them contain protein richer in methionine than the beans that are now extensively used. Other seed-bearing plants could usefully be surveyed in a similar manner because methionine deficiency is a common defect of seed proteins, but there seems to be no reason to think that plants operate under a general constraint on their use of methionine. It is relatively abundant in the seed protein in some varieties fo maize (Fowden and Wolfe, 1957).

Micro-organisms are being intensively studied for use as animal fodder. Many of them would probably be useful human foods if grown on molasses and some other wholesome agricultural by-products. This research will offer scope for considerable biochemical ingenuity, because micro-organisms differ in their ability to use different carbohydrates and agricultural by-products contain a variety of carbohydrates. Efficient production will depend on making the correct matches.

Clearly, there are many problems worthy of investigation : equally there are many reasons why they are not investigated. The search for novel protein sources, or indeed for any new types of food, is often regarded as a job for industrialists rather than academic scientists. Since the time of Hippocrates it has been obvious that food and health are closely connected. Evidence is now accumulating that it would be cheaper (to put the matter on the crudest basis) to feed people better, and so prevent much disease, rather than rehabilitate the patient after disease has developed. Nevertheless, developing countries put more effort into cure than prevention, and medical services are financed by the government, whereas food supplies are left to private enterprise. These things seem anomalous.

Unwillingness to adopt radical research policies on the food supply depends in part on the assumption that the position will automatically improve, and that

all will be well if existing methods are applied more efficiently and over a greater area. The tenuous rational basis for this assumption is that conditions in Britain are now tolerable, whereas 150 years ago they were similar to those in much of the developing world today. But the circumstances are not comparable. British prosperity depended on a world market for the products of the Industrial Revolution, and there were few competitors in it. There was abundant under-exploited land overseas from which food could be imported and, as Eric Williams (1944) cogently argues, there was the slave trade. Developing countries have none of these advantages. They will have to produce the bulk of their own food and, with poor communications, they will have to rely on medium scale production units, using local resources to satisfy local needs.

Some support for the idea that the food supply will soon become adequate everywhere, comes from the "green revolution." Valuable as this "revolution" is, it must be borne in mind that what it has produced so far is more maize, rice and wheat—all crops that are deficient in portein. Its success could abolish hunger but replace it by malnutrition : a step forward rather than a final solution. The "green revolution" started when expert research in tropical countries, which had hitherto been devoted almost exclusively to cash-crops for export, was directed towards food crops. This paper is a plea that its momentum should be maintained and that attention should not be limited to cerel seeds.

The "green revolution" illustrates an important principle in research. Progress depends as often on the realisation that a generally accepted idea is false, as on the observation of a new phenomenon. A wise Taoist aphorism runs "when you can see a reason for doubting, at a point where people have not hitherto doubted, then you are making progress." The development of short-strawed wheat and rice could not start until plant physiologists had got rid of the illusion that grain yield depended mainly on pre-existing leaf : it depends rather on new photosynthesis in the neighbourhood of the grain. This was observed many years ago but practical use was not made of the information. There have been many other examples of delayed application caused either by illusion or lack of imagination. Belief in the more-or-less magical virtues of dung delayed the use of "artificial" fertilisers for 60 years ; unwillingness to accept clear evidence that part of the nitrogen requirement of ruminants can be met by urea delayed its use by 40 years ; and belief that animal proteins are of necessity superior to any plant protein now interferes with the more widespread use of plant proteins. Jensen (1967) gives some other examples.

The "green revolution" also illustrates a disquieting feature of the academic and agricultural scene. It was not brought about by scientists born in the

countries that need extra food—they were busy with more remote and impractical aspects of plant physiology. It could indeed be regarded as "scientific colonialism" perpetrated in a good cause. One reason for this, which is regularly commented on and deplored, is the tendency of scientists, returning to a developing country after a period of training elsewhere, to try to continue with the line of work they had been following in the affluent country. A more serious reason is lack of official appreciation of agricultural and other biological potentialities. Students follow this bad example: fewer than 5% study agriculture in the universities in developing countries or in the universities abroad to which they go for further training. For as long as villages are neglected and governments think of development in terms of urban industrialisation, this attitude of the students is reasonable. They regard education as a way to escape from village life—not to improve it.

The tendency to hold agriculture, and even science in general, in low esteem is not confined to developing countries. According to the US President's Science Advisory Committee report on "The World Food Problem" (1967), scientists engaged in agriculture in the USA are paid less than any other group of scientists, and in the British civil service an administrator is seven times as likely to get £5,000 a year as is a scientist (Profitt, 1967). But things may be changing. The Minister of Agriculture of one developing country was recently quoted as saying "Now I can get the Finance Minister to return my phone calls."

A sound principle governing research in developing countries is "if the work could just as well be done in an affluent country—it should be done there." The laboratory space and human skill of developing countries will produce the most useful and original results when applied to problems peculiar to these regions. But the final presentation must be complete and unassailable. There have been enough hurried and unconvincing "preliminary communications"; what is needed is thorough investigation so that there will be no further argument on the matter. Young scientists are sometimes unwilling to undertake such thorough work, because after several years they will have only one paper to show for it whereas the same amount of effort put into routine research might have produced, or at any-rate permitted sub-division into, a dozen papers. Any tendency in a University to assess merit by the number of publications must be roundly condemned. Any fool can split a piece of work into little bits and get them published separately, only an able scientist can produce a complete and unassailable statement. That proposition has universal application; it has nothing to do with the state of development of a country.

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BIOCHEMISTRY AND SPECTROSCOPY

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Success in the isolation and characterization of natural products presents biochemistry with a great and growing range of problems. It is not merely that the number of naturally occurring organic compounds is so large—one only has to think of the alkaloids—there is also immense variety and complexity in structure.

Newly-described natural products are merging almost daily, from micro-organisms, terrestrial plants, animals and aquatic organisms of all kinds. There is every prospect that the flow of new substances will continue and accelerate.

The reasons behind this flood of compounds inclue (*a*) improvements in methods of isolation, such as different sorts of chromatography (*b*) better ways of determining molecular size and (*c*) advances in methods of ascertaining structure.

Some of us began our researches in the 'nineteen-twenties' when it was necessary to isolate enough of a new compound for 'macro' C, H and N determinations and for carrying out cryoscopic or ebullioscopic molecular weight determinations. Oxygen was obtained by difference! Ultraviolet and infrared absorption spectra could only be measured laboriously and slowly, while the corpus of knowledge was often too small for more than tentative interpretations. Structural studies still depended on classical methods of degradation, confirmed by synthesis.

Today, thanks to theoretical as well as instrumental advances, both on very wide fronts, the position has been transformed. High resolution mass spectroscopy permits molecular weights to be ascertained—in most cases unequivocally and with fantastic accuracy so that trustworthy empirical formulae emerge. Moreover, fragmentation patterns make a considerable contribution towards fixing structures. Ultraviolet and infrared spectra are now measured routinely using modern recording instruments and in each case a large body of classified data is available. Chromophoric groups and identifiable vibration frequencies help to define molecular architecture. Nuclear magnetic resonance spectra, particularly proton magnetic resonance spectra, not only assist in recognising individual types of proton linkage $\underline{CH}$, $\underline{CH_2}$, $\underline{OH}$ etc. but do so quantitatively, indicating the number of such links in a molecule. Physical methods also permit the recognition of *cis* or *trans* forms and optical rotatory dispersion or

circular dichroism afford further diagnostic assistance. Biochemistry, faced with new compounds having defined structural characteristics, tends to be concerned with some of the following matters :—

(*i*) *Biosynthesis.* Whether the compound is derived for example from a plant, an animal or a micro-organism its origin poses many problems. Biochemists may devise experimental approaches using isotopically-labelled likely precursors so that intermediates may be isolated and characterised, using *inter alia* several spectroscopic methods.

(*ii*) *Function.* Many new compounds are isolated because a biological effect has been discovered first. Monitoring by bio-assay, applied classically in vitamin studies, is now evident in research on pheromones. In other areas compounds have been isolated without the help of any biological response and functions have to be looked for. Antibiotics have been 'screened' and useful properties revealed but the study of function *within the source organism* is only in its early stages. It should be noted that some antibiotics are known to act as specific inhibitors, *e.g.* at defined stages in the respiratory chain, and such compounds may be more valuable as research tools than as therapeutic agents.

(*iii*) *Metabolism.* Ingested compounds undergo change within living organisms, *e.g.* animals can 'detoxicate' foreign substances in the liver (although the metabolism can sometimes result in *increased* toxicity).

(*iv*) *Pharmacological properties.* New substances may possess 'beneficial' effects but before they can be brought into wide use they need to be tested for toxicity, carcinogenicity, teratogenicity and mutagenic potency.

(*v*) *Detailed modes of action.* Biochemistry is now rich in arrays of metabolic patterns and once a biological property has been defined as due to a new substance the evidence needs to be fitted into a varified metabolic scheme or schemes.

Analytical chemistry has reached a new level of sophistication and very small amounts of 'active' substances can often be determined. The implications for biochemistry are very broad. Two may be mentioned here, first that analytical *biochemistry* must be kept up to a similar standard and second that the *selection* of problems for biochemical study becomes increasingly critical when programmes could be enlarged almost indefinitely.

The above lines of thought can be illustrated by the role of spectroscopy in different phases of research particularly on unsaturated compounds.

1. *Poly-enes, poly-ynes and poly-enynes*

(*a*) The ultraviolet absorption spectra of linear conjugated poly-enes $R-(CH=CH)_n-R^1$ show peaks near 220nm. ($\epsilon_{max.}$ *ca.* 20,000) when n=2, and as n increases the absorption is displaced to longer wave-lengths, *e.g.* to 330, 340, 360 and 372 nm. for the hexa-ene (n=6). A carboxyl group has an additional bathochromic effect if conjugated, *e.g.* $CH_3CH_2CH_2(CH=CH)_2COOH$ shows $\lambda_{max.}$ 260 nm. whereas the insulating effect of CH_2 is clear :

$$CH_3CH_2(CH=CH)_2CH_2COOH\ \lambda_{max.}\ 228nm.$$

There is much information on the spectra of conjugated poly-enes and it falls into a clear pattern.

(*b*) The spectra of poly-ynes present a similarly coherent picture. The family of compounds

$$CH_3-(C\equiv C)_n-CH_3$$

exhibits in each member one set of weak absorption bands and a second set of very strong bands. The strong bands occur at shorter wave-lenghts, *e.g.* the dimethylpenta-yne shows a very strong peak at 260.5 nm. (ϵ352,000) with subsidiary peaks at shorter wave-lengths with separations *ca.* 2000–2300cm.$^{-1}$. The weak peaks, showing similar separations, occur between 300 and 394nm. with ϵ values 100–500.

The hexa-yne and hepta-yne spectra are basically similar, the location of each of the two groups of bands showing a bathochromic shift for an increaes in *n*. The intense bands show big increases in $\epsilon_{max.}$ as *n* increases but the weak long-wave bands do not. The positions, intensities and separations (Δcm.$^{-1}$) are all diagnostic of the chain length of poly-yne chromophores.

(*c*) Compounds that contain conjugated olefinic and acetylenic groups exhibit similar resolved absorption spectra. Sufficient compounds of known structure have been examined to allow *x*, *y* and *z* to be identified from the spectra of new poly-enynes

$$R-(CH=CH)_x-(C\equiv C)_y-(CH=CH)_z-R$$

(Bohlmann 1952, 1953*a*,*b* ; Hausser *et al.* 1935 ; Nayler and Whiting 1955 ; Jones *et al.* 1960).

2. *Unsaturated fatty acids*

Unsaturated acids with two, three or four conjugated double bonds are 9, 11-linoleic acid ($\lambda_{max.}$ *ca.* ϵ 32,500), α-elaeostearic acid ($\lambda_{max.}$ nm. 262, 271.5, 283, ϵ36,200, 48,100, 37,000, in cyclohexane) and α-parinaric acid ($\lambda_{max.}$ nm. 293, 305, 319, ϵ 50,3000, 77,300, 69,100 in cyclohexane). The displacements on the wave-length and intensity scales are consistent and diagnostic. Simi-

larly the spectrum of stillingic acid fits its structure as a diene conjugated with the carboxyl group. Ximenynic acid (octadec-11-en-9-ynoic acid shows the expected 229nm. peak. Erythrogenic acid (octadec-17-ene-9, 11-diyne) shows in ethanol three peaks at 227, 228 and 252nm. (ϵ 370, 344 and 120) respectively. Capillene $CH_3-C\equiv C-C\equiv C\ CH_2C_6H_5$ shows peaks at 239 and 253nm. (ϵ 537, 427) the absorption due to the phenyl group being masked and the CH_2 interrupting conjugation. The diyne group in erythrogenic acid is similarly insulated from the carboxyl group by CH_2 groups. Bolekic acid is an octadeoc-?-ene?? diynoic acid which has peaks at 227, 240, 253-267, 282.5nm. (ϵ 3060, 6300, 11,700, 17,400, 13,600). Isanolic acid (8-hydroxy-14-ene-10, 12-diyhoic acid) has very similar maxima so that bolekic acid must contain the chromophore

$$-CH=CH-(C\equiv C)_2-$$

but the two acids may not have the same *cis* structure.

Aethusin $CH_3-CH=CH-(C\equiv C)_2-CH=CH-CH_2-CH_3$ from *Aethusa cynapium* L shows the following spectrum :

λ_{max} nm.	249,	266	277,	295.	313,	335
$\epsilon_{max.} \times 10^{-4}$	3.16	2.69	1.66	2.85	3.98	2.85

The alcohol aethusanol-13 shows a very similar spectrum.
(Pitt and Morton 1957 ; Yamaguchi 1970).

Bu'Lock *et al.* (1955) measured absorption spectra for the antibiotic nemotin and its derivatives. Nemotin is an unsaturated lactone :

$$HC\equiv C-C\equiv C-CH=C=CH-CH\begin{matrix}CH_2-CH_3\\ |\quad\ |\\ O\!-\!-C=O\end{matrix}$$

which yields nemotinic acid

$$HC\equiv C-C\equiv C-CH=C=CH-\underset{\displaystyle OH}{\underset{|}{CH}}\ CH_2-CH_2-COOH$$

and on treatment with alkali nemotin A

$$HC\equiv C-(C=C)_2-CH=CH-CH_2-CH_2-COOH$$

The absorption peaks are as follows :—

Nemotin	$\lambda_{max.}$ nm.	208.5	236.5	249	278					
	$\epsilon_{max.} \times 10^{-3}$	44.7	6.17	10.5	12.3					
Nemotinic acid	$\lambda_{max.}$ nm.	209	237	249.5	278.5					
	$\epsilon_{max.} \times 10^{-3}$	44.7	5.62	10.0	12.3					
Nemotin A	$\lambda_{max.}$ nm.	204	211	230	241	258	272	288	307	328
	$\epsilon_{max.} \times 10^{-3}$	26	23	59	85	28	6	12	15.5	11

The spectrum of nemotin is like that if isanolic acid, an ene-diyne and there is little change when the lactone group is opened up. Infrared spectra reveal the presence of an allene group C=C=C (1960cm. $^{-1}$). Alkali isomerisation produces an ene-triyne chromophore also seen in an epoxide from *Artemesia pontica*.

Several antibiotics are known (cryptocidin, flavacid, mediocin, fradicin) which contain a conjugated hexaene chromophore and each of them shows a spectrum with three prominent peaks at *ca.* 340, 35.7 and 378nm. Antibiotics with a heptaene chromophore show three peaks near 360, 380 and 400nm.; they include candidin, candicidin, trichomycin and others.

Fungichromin, obtained from a strain of *Streptomyces cellulosae* has a formula $C_{35}H_{50}O_{13}$, and exhibits ultraviolet absorption indicating the persence of a conjugated penta-ene grouping. When the antibiotic is treated with sodium carbonate and oxidised, using sodium periodate, it yields 2-methyl-2, 4, 6, 8, 10-dodecapenta-enedial (*a*) which is oxidised by silver oxide to the corresponding dicarboxylic acid (*b*) or reduced by sodium borohydride to the diol (*c*). The selective absorption of fungichromin closely resembles that of the diol while the dicarboxylic acid shows the red shift characteristic of an extended chromophore and the dialdehyde illustrates how this shift is carried further (Cope *et al.* 1958, 1962).

Lagosin (Ball *et al.* 1957) has a formula $C_{35}H_{56}O_{13}$, and like fungichromin this substance has a macrocyclic ring within which is the same chromophore (Dhar *et al.* 1964). The same absorption spectrum is shown by filipin (deoxylagosine) and by pentamycin (Umezawa and Tanaka 1958).

Penta-ene absorption maxima

Fungichromin	$\lambda_{max.}$ nm	311	322.5	338.5	357	
	$E^{1\%}_{1cm.}$		1420 – 1450		*ca.* 99,000	
Pentamycin	$\lambda_{max.}$ nm.	308 – 313	332	338	356	
	$E^{1\%}_{1cm.}$		899	1450	1500	
Diol * (*c*)	$\lambda_{max.}$ nm.	240	319	334	351	
	$\epsilon_{max.} \times 10^{-3}$ *ca.*	3.5	65.5	107	108.5	
Dial (*a*)	$\lambda_{max.}$ nm.	222	284	384	402	
	$\epsilon_{max.} \times 10^{-3}$	7.64	6.2	77	75	
Diacids	$\lambda_{max.}$ nm.	211	270	350	366.5	382.5
	$\epsilon_{max.} \times 10^{-3}$	8.4	4.4	46	86	81

*2—methyl, —2, 4, 6, 8, 10—dodecapentaendiol

Mycomycin (Celmer and Solomons 1952, 1953) has the formula $C_{14}H_{12}O_2$ and despite its evident considerable unsaturation it shows a simple but strong spectrum :

$\lambda_{max.}$ nm.	~256	267	181
$\epsilon_{max.} \times 10^{-4}$	3.5	6.1	6.7

Alkali isomerises mycomycin to isomycomycin which exhibits a much more complex absorption spectrum :

$\lambda_{max.}$ nm.	246	257.5	267	287,5	305.5	324	347
$\epsilon_{max.} \times 10^{-4}$	2.4	5.8	11	1.4	2.7	4.1	3.4

The infrared absorption of mycomycin has peaks at 3180cm.$^{-1}$ (≡CH) 2200cm.$^{-1}$ (−C≡C− disubstituted), 1930cm.$^{-1}$ (C=C=C) and 1730cm.$^{-1}$ (COOH). Isomycomycin has lost the 1930cm.$^{-1}$ peak but the 1730cm.$^{-1}$ peak persists. The very high value ϵ=110,000 for the 267nm. peak in isomycomycin points to an ene-triyne structure.

Transdehydromatricara ester (Bohlmann and Mannhardt 1955).

$$CH_3-(C\equiv)_3-CH=CH-COOCH_3$$

shows a similar spectrum :

$\lambda_{max.}$ nm.	244.7	255	268	283.5	300.8	320-374	343.5
$\epsilon_{max.} \times 10^{-4}$	7.18	10.62	8.6	1.5	3.2	4.5	6

The allene group (Celmer and Solomons 1953) does not function like a conjugated diene :

	$\lambda_{max.}$ nm.		$\lambda_{max.}$ nm.
$RCH=C=CH_2$	170	$RCH=CRH$	185
$RC=C=CH_2$ (COOR)	210-215	$RC=C=CH_2$ (COOR)	212-214
$Ph_2C=C=CPh_2$	267	$Ph_2C=CH_2$	250
$C_{10}H_7.CH=C=C=CH.Ph$	298	$C_{10}H_7CH=CH_2$	295

Mycomycin has the structure :

$$\overset{13}{C}H\equiv\overset{12}{C}-\overset{11}{C}\equiv\overset{10}{C}-\overset{9}{C}H=\overset{8}{C}=\overset{7}{C}H-\overset{6}{C}H=\overset{5}{C}H-\overset{4}{C}H=\overset{3}{C}H-\overset{2}{C}H=\overset{1}{C}H\ \overset{14}{C}H_2COOH$$

and it exhibits two separate chromophores an ene-diyne and a tri-ene acting additively.

Isomycomycin with the structure

$$CH_3-\overset{13}{C}\equiv\overset{12}{C}-\overset{11}{C}\equiv\overset{10}{C}-\overset{9}{C}\equiv\overset{8}{C}-\overset{7}{C}H=\overset{6}{C}H-\overset{5}{C}H=\overset{4}{C}H-\overset{3}{C}H=\overset{2}{C}H\ \overset{1}{C}H_2COOH$$

is a conjugated triyne-diene.

The central carbon atom of the allene group insulates the two chromophores of mycomycin but it happens that they have similar peaks. Another triyne-diene

$$CH_3-(C\equiv C)_2-(CH=CH)_2\,CH_2\,CH_2\,CH_2\,O\,COOCH_3$$

has been obtained (Bohlmann *et al.* 1960). It shows peaks at :

λ_{max} nm.	258	268.5	288	305	325	348
$\epsilon_{max} \times^{-10}$	5.63	11.45	14.6	27.5	4.16	3.39

Oroshnik *et al.* (1955) pointed out that the antibiotic flavacid had in the visible region the same (qualitative) absorption as 2, 15-dihydrocrocetin and the spectrum of the antibiotic nystatin reproduced that of α-parinaric acid. The absorption spectra of certain carotenoid precursors contain the same chromophores.

It will be seen that antibiotics with poly-ene, poly-yne or ene-yne chromophores possess in their ultraviolet spectra and sometimes their infrared absorption spectra very useful labels for following biosynthetic pathways, metabolic fates and to some extent modes of action.

It is interesting to recall that provitamins D (7-dehydrocholesterol, ergosterol) are conjugated dienes and the presence of two double bonds in sterol ring B makes a very characteristic chromophore. Exposure to ultraviolet light of the wave-lengths absorbed initiates the sequence of events which gives sise to vitamin D. Cholecalciferol is a conjugated tri-ene in an opened ring system and the observed change in absorption is today less surprising than it was originally. The later work of Velluz (1955) established that irradiation of provitamin D resulted in a previously unsuspected intermediate previtamin D. This has an (unresolved) absorption spectrum with λ_{max} 262nm., ϵ_{max} 9,000 whereas vitamin D has similar absorption but with a higher extinction coefficient (ϵ_{max} 18,300). The infrared absorption of the vitamin includes a peak at 900cm.$^{-1}$ attributable to its exocyclic double bond but this vibration is not shown by previtamin D.

Tachysterol (λ_{max} 281nm., ϵ_{max} 24,600) has a tri-ene system with one double bond in each of two rings ; moreover in the infrared it shows a band at 955cm.$^{-1}$ indicative of a *trans* structure. Koevoet *et al.* (1955) thought that previtamin D_3 was a 6,7-*cis* isomer of tachysterol and confirmed the hypothesis by iodine catalysed isomerisation of previtamin D_3 to tachysterol. Moreover Velluz found that light effected (a partial) isomerisation of previtamin D_3 to tachysterol. In the last year or two vitamin D has been found to be a precursor of a hormone 1,25-dihydroxycholecalciferol made in the kidney (Kodicek 1969*a*, *b* ; Fraser and Kodicek 1970 ; Lawson *et al.* 1971 ; DeLuca 1969 ; Melancon

and DeLuca 1970 ; Holick *et al.* 1971). Students of absorption spectra in the ultraviolet and infrared regions can learn much by pondering over the vitamin D story (*cf.* Fieser and Fieser 1959). The spectra of steroid dienes, tr-enes and tetra-enes depend on positions of olefinic links in the tetracyclic molecules. The positions and intensities of absorption peaks are of considerable diagnostic value.

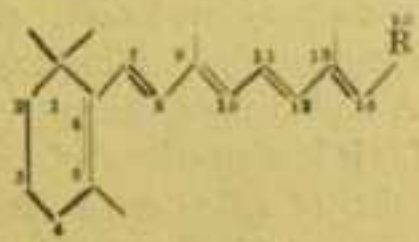
If vitamin D concerns in the main diene and tr-ene chromophores the carotenoids include extended chromophores with some 10, or 11-conjugated double bonds. In the new volume 'Carotenoids' edited by O. Isler, Vetter *et al.* (1971) contributed a large collection of spectroscopic data. An immense amount of work on the ultraviolet and visible absorption spectra has been reduced to order. This applies also to vitamins A_1 and A_2 and their derivatives. Infrared spectra have played a minor but very useful part in detecting acetylenic and allenic groups, thus the 1928cm.$^{-1}$ peak of fucoxanthin indicates an allenic group and the 2170cm.$^{-1}$ peak of alloxanthin is characteristic of an acetylenic carotenoid.

N.m.r. spectroscopy has been applied with vigour in the carotenoid field and the systematised information now available has helped to make the investigation of structure in a new carotenoid almost a routine. A point of special interest is that recent work on n.m.r. spectra of carotenoids allows comparisons to be made of the performances of the 60 MHz, the 100 MHz and the 220MHz instruments—a matter of importance when money for equipment is in short supply !

The significance of *cis-trans* isomerism in the carotenoid field was first elaborated by Zechmeister and extended later (*see* Barber *et al.* 1962 ; Weedon 1971). The 220 MHz instrument permits for many poly-enes a resolution of the olefinic part of the spectrum so that the position of almost every proton can be ascertained. This is significant point for vitamin A isomers and particularly so for the *cis-trans* forms of retinaldehyde. The 11-*cis* forms of the aldehydes of vitamins A_1 and A_2 give rise to visual pigments.

Table

Chemical shift for retinol and retinaldehyde



All-*trans* retinol Retinaldehyde

		All-*trans*	11-*cis*		9,13-di*cis*	
Olefine proton				*cis-trans*		*cis-trans*
	7	6.14	6.36	6.32	6.36	
	8	6.09	6.17	6.14	6.68	+0.54
	10	6.08	6.19	6.54	+0.35 6.16	
	11	6.60	7.15	6.69	−0.46 7.16	
	12	6.27	6.37	5.92	−0.45 7.25	+0.88
	14	5.67	5.97	6.07	+0.10 5.87	−0.10
CH_2	15	4.29				
CH_3	9	1.97	2.05			
CH_3	13	1.85	2.32			
Ultraviolet absorption	CHO	10.09	10.10		10.27	+0.17
λ_{max} 325nm.		381	376.5		37.3	

In this paper the stress has been on *conjugated* poly-enes and poly-ynes. Unconjugated poly-enes are however important in many ways. Thus the higher polyprenols like dolichols and the alcohols solanesol and the newer castaprenols etc. are transparent to ultraviolet light but can profitably be studied by other spectroscopic methods. *Cis-trans* isomerism is revealed by infrared and n.m.r. spectra. These alcohols are carriers in biosynthesis of complex carbohydrate derivatives. The polyprenyl side-chains of plastoquinones, ubiquinones and menaquinones are present in molecules needed for photosynthesis, electron transport and vitamin K activity (Morton 1971).

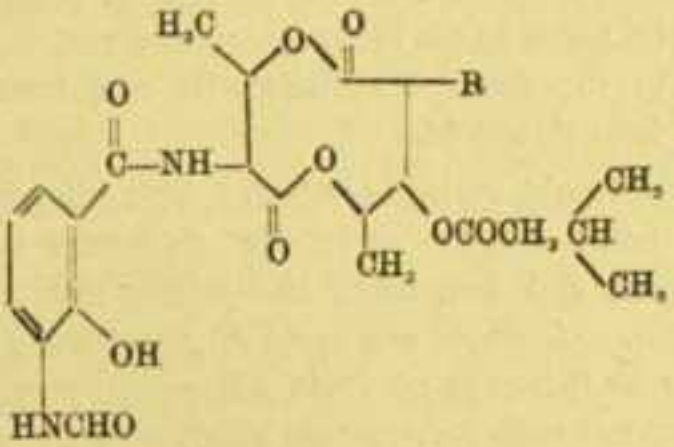
Numerous examples occur where mass spectra fragmentation patterns have greatly helped in the elucidation of structure of such poly-unsaturated compounds. Problems of biosynthesis in the carotenoid field have been the subject of highly successful effort. The whole topic is reviewed in detail by Goodwin in Isler's very recent volume. There is nothing to be added here except that some recent developments illustrate how new and intriguing issues are arising as research proceeds.

Batra (1967*a*, *b*) observed that a non-photosynthetic bacterium *Mycobacterium marinum* produced cartoenoids only when illuminated. This photoinduction is made up of a temperature-independent, oxygen-requiring photochemical phase followed by dark reactions needing oxygen but not light. It was also found that antimycin A could induce carotenoid synthesis in the organism in the absence of light. The induction effects of the antibiotic and of light were additive so that different sites of action could reasonably be postulated.

Antimycin A is well-known as an inhibitor in the electron transport system of aerobic organisms including yeasts, fungi and animals (Rieske 1967) the

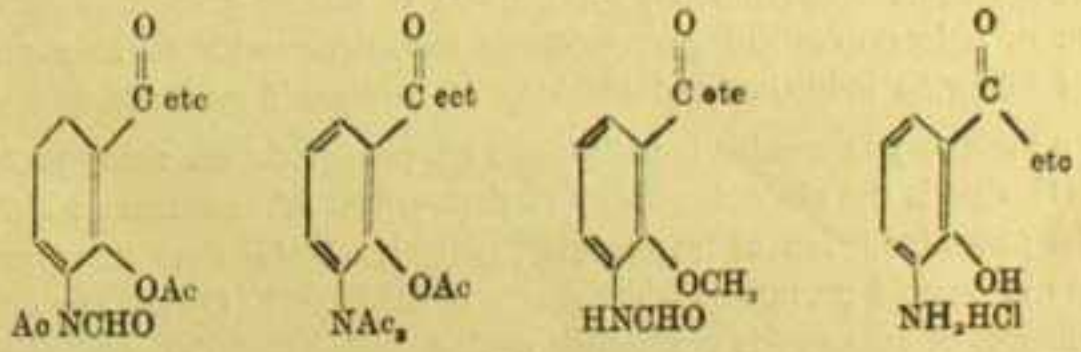
effect being located at the cytochrome level (Chance 1958). Antimycin A exists as a complex with four components all of which inhibit electron flow. A similar effect is also obtained with 2N-heptyl-4-hydroxyquinoline-N-oxide but that compound does not induce carotenoid biosynthesis in *M. marinum*.

Batra *et al.* (1971) made a study of the relationship between structure and activity in the antimycin-induced carotenoid synthesis. Antimycin A consists of four compounds each of which was as active as the antimycin A complex as a whole (optimally at 40$_\mu$M) and in each case light enhanced the effect additively. [The components differ in respect of the alkyl substituent in the dilac tone ring.]

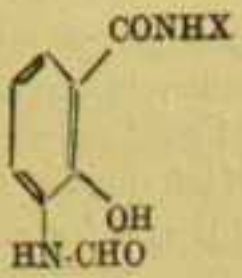


R = Hexyl Antimycin A$_1$

R = Butyl Antimycin A$_2$



The chromophoric portion of the antimycin molecule is the aromatic 3-formamidosalicylamide moiety :



but a dozen synthetic compounds differing in respect of the group X (hexyl, octyl, decyl, phenyl, benzyl, etc.) all failed to induce carotenoid biosynthesis from which it was concluded that the dilactone ring was indispensable. Furthermore antimycin diacetate and antimycin triacetate were very active—although they may have been unstable at the pH 1 (8.0) used. Deformylation did not reduce the activity so that the N-formyl group was not essential for inducing carotenoid biosynthesis although it is indispensable for the inhibitor effect in electron transport.

The methyl ether was synthesised and its purity checked by nuclear magnetic resonance (the downfield resonance due to the phenol proton was replaced by an intense signlet at 6.1. due to the methoxy protons) and it failed to induce carotenoid synthesis in the dark. Surprisingly it stimulated light-induced cartenoid synthesis at least three-fold.

The stimulus to carotenoid synthesis effected by antimycin A displays a lag period of about 4 hours, a source of nitrogen is needed and some known inhibitors of transcritpion and translation also inhibit carotenoid production (Batra *et al.* 1968, 1969). Moreover antimycin A stimulates protein synthesis in *Tetrahymena pyriformis* (Elson *et al.* 1970). Hence it seems probable that both light and antimycin A induce carotenoid production by derepressing the genetic sites resulting in the synthesis of mRNA and the enzymes needed for carotenogenesis. The lag period observed with the methyl ether plus light could have been due to preliminary synthesis of carotenoid-producing enzymes. Addition of chloramphenicol gave evidence consistent with *de novo* protein synthesis following induction and blocking of carotenoid synthesis.

When these very interesting investigations are pondered over many problems arise : (1) what is the absorbing entity in dark-grown *M. marinum* which initiates the photo-induction of carotenoid production ? (2) does the organism make any carotenoid precursors when grown in the dark ? (3) are any colourless precursors of carotenoids synthesised during the lag period ? (4) inasmuch as a number of enzymes are needed for biosynthesis of the carotenoids and their precursors do both light and antimycin derepress several genetic sites ? These complications are doubtless evident to the workers concerned but it will be noted that the implications are increasingly biochemical.

This review illustrates how spectroscopic techniques have contributed to a transformed approach to structure, biogenesis and function. Biochemists not only need fine instruments and good technicians, they also need to master the essential parts of a vast body of information that has been patiently gathered and reduced to order. The time is ripe for penetrating biochemical studies.

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IMPROVEMENT OF NUTRITION DURING POSTWAR ECONOMIC DEVELOPMENT IN JAPAN

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The Second World War drove the Japanese people to the edge of starvation and occurrence of a large number of malnutrition patients. But after the War, the food supply recovered to a normal status in a relatively short period of time, and, moreover, some improvement in the eating habits has been made quite smoothly. At present we see very few cases of deficiency of certain nutrients. We can see the change in food supply per capita per day in calories since 1930 according to the data provided by the Ministry of Agriculture and Forestry. We see 60% of total calories was supplied by rice before the War. After the period of low calories during and directly after the War, the calorie supply was recovered to the pre-war level in 1955. The rate of calories supplied by rice, however, did not reach the pre-war level, and it has begun to decrease soon. At present rice gives 40% of the total supply of calories. On the other hand, the supply of other cereals has increased a little compared with the pre-war level. The chief amount of these cereals consists of wheat in a form of bread, the use of which has been recommended through the school lunch program practiced in the whole country. The distribution ratio of calorie supply by cereals is decreasing year after year, and it is now about a half of the total calories. During and directly after the War potatoes and sweet potatoes were consumed in large quantities, but this trend has been decreased in the recent ten years, too. Despite the decreased consumption of rice and potatoes, the total calorie intake is increasing slightly every year because of the increased consumption of starch, sugar, fats and oils, and other foods. Pulses remain almost constant, but the supplies of vegetables and fruits have increased. Fish and shellfish remain constant, but the supplies of milk, meats and eggs have greatly increased in recent years.

As for the protein supplies, a remarkable increase occurred after the War. The net protein supply per capita per day was 54-55g before the War, and 36g directly after the War, that is, in 1946. In 1950-51 it recovered the pre-war level and after that, it increased year after year. It is now 75g, and this increase is largely due to the increased supplies of meats, eggs, milk and milk products.

The distribution ratio of animal protein in the total prtein in the pre-war was was 16%, whereas it has been increased to 40% at present.

The national nutrition survey in Japan were started in 1948. In 1952 the Nutrition Improvement Law took effect, and since then, the Ministry of Health and Welfare has been in charge of the Annual Nutrition Survey. In practice, doctors and dietetians take records of food intakes for five consecutive days of more than 16,000 families, representing 1/2000 of the total population of Japan. In the nutrition survey data we see the general trends of the improvement in nutritional intake of the Japanese people during the past 20 years, although some slight discrepancies are observed between these data and the food supply data given by the Ministry of Agriculture and Forestry. As for the nutrient intake, the data on protein are in a good agreement. The data on fat consumption show the increase since 1960, and it has reached to 18% of the total calorie intake, 40% of which comes from animal fats. Carbohydrate consumption is decreasing, and the increased consumption of fats and proteins contributes to the increase of the total calorie intake. The intake of calcium is 529mg, and that of vitamin A is 1,421 I.U. (as retinol) in 1968. The intake of these two nutrients is still below the Recommended Dietary Allowances for the Japanese. Thiamine (1.10mg) and riboflavin (0.96mg) are also short when the loss due to cooking is taken into account, but in these data neither intake of fortified cereals (rice and wheat) nor use of vitamin tablets is included ; both of them are very popular at present.

I would like to mention the change of the weight and the height of the Japanese people. We have the data of Japanese school children and students given by the annual physical examinations conducted by the Ministry of Education since 1900. For the values of 6-14 years of age, the data can be considered as representing the national level because of the compulsory education system. For the values of adults we can take the data given by the National Nutrition Survey. The height and the weight of the Japanese youth increased gradually between 1900 and 1935. At the end of the War, however, the build of the Japanese youth was as poor as the build in the year 1900. After the War, the build of these age groups recovered rapidly to the pre-war level, and the marked trend of increase in growth is still continuing at present. In the physique of adults we can also notice the increase in height and weight. The weight of the group of 19-20 years of age increased steadily in males, while a plateau of the weight curve is noticed in females in the same age group, probably showing the results of the individual weight control. When the mean height of the Japanese people of 1948 and that of 1968 are compared, the values of 1968 are heigher in all ages than those of 1948 in both males and females. When

the mean weight of 1948 and that of 1968 are compared, the same is true except for the age group around 20 in females, and the weight curves do not go up in the older age both in 1948 and in 1968.

We do not think that the above-mentioned increase of the physique is entirely due to the improvement of nutrition, but it was proved that the positive correlations existed between the increase in height or weight and the intake of fat, animal protein, calcium, riboflavin and vitamin A. Vegetable protein and carbohydrate showed negative correlations.

We shall turn the topic to the relation of the food intake and the economic circumstances. The living expenditures of the Japanese people are reported by the Bureau of Statistics, Office of Prime Minister of Japan, as the Annual Reports of the Family Income and Expenditure Survey, the data of which are the means of 8,000 households, selected at random on a nationwide base. In the reports we can see the gradual decrease of Engel Coefficient from 63% in 1948 to 34% in 1970. The distribution ratio of cereals in the total food expenditures has been decreasing every year from 39.1% in 1950 to 15.9% in 1970. When the relation between the average monthly disbursement and the food intake was sought, it was observed that, the lower the disbursement, the higher was the cereal consumption. The protein intake increased as the monthly disbursement increased. The consumption of animal protein was responsible for this difference. The fat intake increased with the increase of disbursement, and this is true for both animal and vegetable fats.

These differences among the disbursement groups, as well as the differences between urban and rural areas are becoming smaller now, but we have still some important nutritional problems such as anemia among females at present. Incidence of regulatory obesity is increasing not only in adults but also in children.

I would like to compare the situation of food supplies and the cause of death by diseases between Western Countries and Japan. The food supply data were taken from the Statistical Yearbook of United Nations, and the data on the causes of death were taken from the WHO World Health Statistics Annals. Compared with the food consumption in Western Countries, the consumption of cereals and pulses are high and that of total calories, sugar, meat, milk, fat and oils is still low in Japan. As for the cause of death, apoplexy is the highest cause of death in Japan, and the death rate caused by this disease is higher than that in Western Countries. On the other hand, the death rates due to coronary heart disease and diabetes mellitus are very low compared with those in Western Countries. However, the incidences of diabetes mellitus, regulatory obesity, gout and dental caries are increasing.

At the end of this paper I shall add a few words about the administrative activities which were carried into effect and contributed to the nutritional improvement in Japan. First I should acknowledge the help given by the Organizations of the United Nations such as UNICEF, and the United States and other countries directly after the War. There are four laws enacted after the War in Japan : Nutritionist Law, Nutrition Improvement Law, School Lunch Law and Cooks Law. The number of the training schools for nutritionists is 258, and the number of licensed nutritionists is 773,000. As for the school lunch program, the number of the boys and girls receiving the school lunch is 98% of the total primary school children and 83% of the secondary school pupils. As for the enrichment of foods, 30% of the consumed rice in Japan is enriched with thiamine and riboflavin. Wheat flour, bread and noodles are very often enriched with thiamine, riboflavin, calcium and lysine. Soybean paste is enriched with vitamin A, thiamine, robiflavin and calcium.

A NEW AREA OF BIOCHEMICAL INTERESTS

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Chemistry is one of the most ancient subjects of study and has had essentially an applied outlook. It has been interested in transmutation in the widest sense of converting less valuable materials into highly valuable ones. It has also been interested in discovering the elixir of life in the widest sense, that is the discovery and preparation of drugs of greatest efficacy. Consequently its twin objectives have been to produce wealth and health for the community. In this work it had to deal with subtle features regarding the internal constitution of matter, subtle secrets that could not be seen, but had to be tested for by special methods. Because of this feature chemistry in early history was frequently mistaken for witch-craft and magic. In more modern times the teaching and understanding of chemistry has been comparatively more difficult. Unless lectures are accompanied by helpful and attractive experimental demonstrations and emphasis is laid on familiar applications, the subject cannot be made attractive. In post-war years the practice of lecture demonstrations seems to have ceased and so also emphasis on applications to realities of life and consequently there is fear that the great popularity that the subject had during the last century and the early part of this century, is fast declining.

Though one of the oldest, chemistry is still one of the youngest in vigour and freshness of outlook. It can never become senile or dull or exhausted. With more effort and more speed put into chemical studies, a faster expanding horizon in revealed with larger scope in every direction. On a rough estimate, in a ten-year period, it gets renovated with a new spirit, with new techniques and new areas of study. Consequently the votaries of chemistry and especially the leaders have to be devoted fully and keep abreast of developments or they will be left behind. They should create the wave and effectively ride it.

Biochemistry as the latest branch of chemistry has become very important in regard to one of the major objectives of chemistry, that is health of human and animal beings. One aspect of it, ascorbic acid (vitamin C), its production and function, was Prof. B. C. Guha's life interest. A line of study following the discovery of ascorbic acid has in recent years made rapid advance. In the thirties, Prof. Szent Gyorgyi, the discoverer of ascorbic acid wrote a small article in *Current Science*, entitled 'Vitamin C to vitamin P.' Therein he narrated how he was led to realise that the commonly occurring group of plant

pigments, called flavonoids, play an important part in maintaining the efficient function of blood capillaries. Considerable literature has subsequently grown up on the use of bioflavonoids, obtained as by-products of the citrus fruit industry, and of rutin, a water soluble glycoside obtained from buck wheat and eucalyptus leaves, for various ailments. The term 'rutin therapy' has been used. A more recent development raises the question of molecular size. The leaves of Gingko biloba, a tree considered by botanists to be a living fossil, contains a number of compounds called biflavonoids that have double the size of normal flavonoids. Extracts of these leaves have been found to have good effect on blood flow in peripheral and cerebral areas and have been marketed as drug. These biflavonoids are also found in many other Gymnosperms common in the mountanious areas of India and can be extracted from them. This study has further taken us on to a large number of flavonoid derivatives that have similar molecular size and are present in various vegetable foods and drugs. The part they play in maintaining human health is important to investigate and utilise. We have recently been studying a very important wood available in South India called Red Sandal (*Rakta Chandan*). It is largely used for art work and for musical instruments and has been used as home remedy in the form of extract or fine paste. Earlier it was also used as a vegetalb dye. Its chemical components are rather complex and baffled attempts by eminent chemists to unravel their chemical constitution for over a century and a half. We have now established that the pigments of the wood belong to a novel category of biflavonoids and determined the details of their main structures. We hope that this will make a vital addition to our knowledge of physiologically active compounds.

TOWARD THE CONQUEST OF MALNUTRITION

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I am happy to have this opportunity of paying my tribute to my friend, late Professor B. C. Guha. I had come into close contact with Professor Guha during the years when we were both on the Nutrition Advisory Committee—he as the Chairman and I as its Secretary. His death at a relatively young age was a great blow to all of us especially to nutrition scientists in this country.

Professor Guha was not only a true scientist but also a great humanist. What particularly impressed me was the breadth of his outlook. While Professor Guha reached great heights in the field of nutritional biochemistry, he also had a remarkable sense of perspective which enabled him to recognise the wide implicatios of the problems of malnutrition in our country and the urgent need for combating them. His horizon was certainly not limited to his own speciality of biochemistry.

I, therefore, felt it would be appropriate to include int his volume which dedicated to his memory an article on the subject "Toward the conquest of malnutrition."

During the years that have elapsed since our independence we have no doubt had some significant achievements to our credit in the field of public health. Thus, malaria has been largely controlled and infant mortality rate has been nearly halved. Unfortunately, however, the nutritional status of our people has shown no significant improvement in the last two decades. There has no doubt been considerable increase in food production in the country, but this has been almost completely offset by the increase in population. The per-caput availability of food supply for the last two decades has remained more or less stationary.

OUR DIETS

The figures for per-caput availability of food supplies, unsatisfactory as they are, tend to under-estimate the problem of malnutrition in the country. There is considerable maldistribution of the available food among different regions of the country and among different socio-economic classes of our people. The income levels in several groups of our population would hardly permit the achievement of even low-cost balanced diets by the families. Our national

average dietary intake of about 2000 calories and 50 g. of protein daily (6 g. of animal protein) itself compares unfavourably with the national average of over 3000 calories and nearly 90 g. of protein daily (over 50 g. of animal protein) of countries like the United Kingdom and the United States of America. Vast numbers of our people are obviously living on diets which provide much less calories and proteins than what these national averages indicate. A country-wide survey carried out recently showed that the diets consumed by young children of the poor income groups in our country provided less than 70% of their actual minimum daily calorie requirements. These diets were also found to be deficient in a number of other important nutrients like vitamin A and iron. Such inadequacies must inevitably result in widespread malnutrition.

Severity of the problem

The magnitude and the full implications of the problem of malnutrition in the country have, however, not yet been fully appreciated. Actual deaths from acute starvation may be few but chronic starvation and milder degrees of malnutrition and undernutrition are widespread and take a heavy toll. More than 80% of our children in the poor income groups are of substandard height and weight and nearly 25% of them show frank signs of nutritional deficiencies. Of these, blindness arising from vitamin A deficiency and severe forms of 'calorie-protein deficiency' represent the most severe manifestations.

A striking indication of malnutrition among our children is the fact that nearly 40% of the total deaths in our country take place in children under 4 years of age, the corresponding figure for the United Kingdom is 4%. Such rapid 'turnover' of the population in this unproductive age period is a serious economic wastage and acts as a disincentive to our family planning programmes. The long-term and permanent effects of malnutrition during the crucial years of development are now beginning to be appreciated. There is growing evidence that malnutrition in early childhood could result in permanent impairment of growth, physical stamina and probably even learning ability.

Enormous amounts are being expended to achieve the minimum objective of primary education for our children. Unfortunately, however, the health and nutritional status of a large proportion of our school children are so poor that a good proportion of this expenditure becomes infructuous. Nearly 15 to 20% of children in our schools show clinical signs of malnutrition and this must be a major factor contributing to the high rates of absenteeism, drop-outs and poor performance.

Nearly 25% of our hospital beds, especially in our children's hospitals are taken-up by cases of frank malnutrition adding to the present scarcity of hospital beds and straining our already meagre public health resources.

Apart from children, malnutrition takes a heavy toll among women of the child-bearing age. Nutritional anaemia is a major cause of maternal mortality in our country. Our maternal mortality rate today stands at 250 per 1,00,000 livebirths as against 25 in United Kingdom.

Nutritional deficiency diseases like pellagra and goitre, which have been eliminated from Western countries where they are today no more than medical curiosities, continue to afflict large numbers of our people.

There can be no doubt that the problem of malnutrition poses the greatest challenge to our administrators, scientists and technologists and on their ability to meet this challenge would depend the health and the well-being of our future generations.

Means of prevention

Our strategy in combating malnutrition must include both long-term measures which would eventually lead to an enduring improvement of the nutritional status of our people as well as specific short-term measures which would immediately serve to ameliorate malnutrition in the weakest and most vulnerable sections of our people. We may briefly review these approaches :

Immediate measures

Specific remedial measures :

Specific remedial measures are essential to control certain major nutritional deficiency diseases which are widely prevalent in our country today. The three major nutritional programmes which fall under this category are : the control of goitre in the extensive Sub-Himalayan belt through a vigorous execution of the programme of iodisation of salt ; the control of anaemia in pregnancy through a well organised programme of distribution of iron and folic acid tablets to pregnant women through the net-work of maternal and child welfare centres in our country ; and the prevention of blindness resulting from severe degrees of vitamin A deficiency through an intensive programme of oral administration of massive doses of vitamin A at six monthly intervals to pre-school children in the Southern and Eastern parts of our country. It is gratifying that our Government has provided budgetary allocations for these programmes and it is to be hoped that they will be effectively implemented. Their implementation does not call for any improvement in the economic standards

and can be achieved under the existing circumstances. The practical administrative and logistical considerations involved in their implementation, however, have to be carefully worked out.

The pre-school child :

From the nutritional standpoint children of the pre-school age group are the most important and the most vulnerable. Some of the worst ravages of malnutrition are to be seen in this age group. Our immediate efforts, therefore, must be directed towards protecting this important section. It is gratifying that the Government of India have in fact made impressive budgetary allocations for the organisation of nutrition programmes among poor pre-school children in the backward areas of the country. The two major questions of practical importance are (*i*) the choice of the dietary supplements to be given to these children to prevent malnutrition, and (*ii*) the means by which pre-school children in rural areas could be reached in large numbers.

It should be obvious that under the present circumstances any large scale programme of feeding of pre-school children must be based on locally available inexpensive foods and not on imported foods or centrally processed foods. The scale of these feeding operations would be such that it would be impossible to base them on a single processed food preparation consisting of two or three selected ingredients. The immense problems of transport, storage and distribution preclude an over-centralised approach to this programme which must, under the circumstances be necessarily decentralised and diversified. Food available within a radius of ten miles from the village could easily form the basis of feeding programmes of children of the village. The Indian Council of Medical Research has worked out a number of nutritions recipes based on such locally available foods, the cost of which may be about 12 paise per day per child. These foods can be processed by methods which are capable of application at the village or the home level.

Today, the cost of transport, distribution, supervision and administration of most feeding programmes for pre-school children represents a considerable proportion of the overall cost of the programme. This can be avoided and real community participation ensured if the village community itself is given the opportunity of, and responsibility for, organising and running the programme. This, however, implies that such feeding programmes for pre-school children should be attempted in the initial stages only in those villages where the necessary leadership and social climate exists.

The organisation of nutrition programmes among pre-school children poses several formidable practical problems. While school children can be collec-

tively reached in large numbers through schools, and while considerable experience with regard to school lunch programmes already exists in our country facilities for reaching pre-school children in large numbers are still very inadequate and large-scale experience in the operation of nutrition programmes among pre-school children has yet to be gathered. The immense administrative and logistical problems implied in nutrition programmes for pre-school children have apparently not been fully appreciated and understood. Till such time this happens, these programmes will proceed unsatisfactorily in spite of impressive budgetary allocations. Our immediate efforts should be directed towards organising the necessary facilities for these programmes at the intermediate and peripheral levels. This does not necessarily imply the creation of new institutions or the appointment of fresh personnel. The existing functionaries at the Block level should be oriented and trained to include nutrition programmes as an important integral part of their activities. Similarly existing institutions like the M.C.H. Centres, the Primary Health Centres, the Mahila Mandals and the Balwadis should be geared for the implementation of large scale nutrition programmes specially directed towards pre-school children. The success of these programmes will ultimately depend upon our ability to harness the existing administrative infrastructure and the institutional framework at the Block level for this purpose.

It would be desirable if the Planning Commission and the Government of India set-up demonstration-cum-training centres for the operation of feeding programmes for pre-school children in three or four selected centres in the country where the requisite leadership and facilities for supervision and evaluation of the programme exist. This will enable us to build up the necessary expertise for organising and implementing the pre-school child feeding programmes on a country-wide scale.

The middle income group :

The main beneficiaries in most of the nutrition programmes at present envisaged are the poorest segments of the population. Considerable degree of malnutrition also exists among other sections, specially, the lower middle classes —children of teachers, petty shopkeepers, skilled workers, artisans and the so-called white collar workers. The scale and quality of the nutrition programmes now being attempted are such that these sections of the population will not be benefited. This is unfortunate because these lower middle classes, in many ways, constitute the backbone of our society. While the poorest group should continue to receive attention, programmes designed to improve the lot of the important middle income groups will yield really impressive divi-

dends. For example, low-cost nutritious biscuits made of inexpensive locally available foods even in village bakeries on co-operative basis, could form the basis of a nutrition programme for this middle income group. Such biscuits stocked in schools, offices and other establishments, and sold to the needy at nominal price, could be one way of reaching this important segment of the population.

Long-term approach :

In the final analysis, satisfactory nutrition can be ensured for our people only through improvement of their economic status, through rising our food production to levels which would meet the increasing needs of our growing population and through restricting the rate of our population growth to levels commensurate with our resources. There can be really no shortcuts to the permanent solution of the problem of malnutrition. The current drive for increased food production and for family planning are steps in the right direction which will eventually make their impact but they cannot bring about any immediate transformation in our nutritional situation.

Recent developments in agricultural technology offer hope of our being able to meet at least our overall foodgrain requirements in the near future. If the necessary inputs are forthcoming we may soon be able to achieve self-sufficiency in cereals. However, this happy situation should not rouse undue expectations and lull us into a false sense of complacency. The conquest of malnutrition implies a lot more than the mere achievement of overall self-sufficiency in foodgrains. The regional, social and economic imbalances which today contribute to maldistribution of available food have to be corrected. The achievement of self-sufficiency in foodgrains together with the correction of these imbalances will no doubt go a long way in correcting the current widespread calorie shortage, but our diets will still stand in need of considerable qualitative improvement. In fact, our deficiency with regard to foodgrains is relatively of a much smaller order than the gaps with regard to other protective food elements like fruits, milk, fish, eggs and meat. Unless these gaps are filled there can be no qualitative improvement in our diets.

It is essential at this point to impart a new nutritional orientation to the so-called agricultural revolution, in order to ensure balanced augmentation of food production in accordance with our nutritional needs. New varieties of foodgrains which are released for propagation should be not only high yielding but also of high nutritive value. This is fortunately being increasingly appreciated.

Apart from prestige cereals like wheat and rice other foodgrains and oil-seeds must receive increasing attention. Millets constitute the staple of the poor segments of our population and deserve special consideration. Since Indian diets are likely to be predominantly cereal based for some years to come it is essential that the dietaries should include reasonable quantities of pulses and legumes in order to provide proper amino acid balance. The pulse-cereal ratio in the present pattern of food production is already unsatisfactory from the nutritional standpoint and unless high priority is accorded to the promotion of pulse production our diets are likely to become even more unbalanced in future. Now that we are in sight of achieving the initial objective of self-sufficiency in foodgrains our agricultural strategy should be oriented towards the promotion of increased production of other protective food elements like fruits, milk, eggs and meat as well.

Today, fortunately, the importance of nutrition is being appreciated at the highest levels in our country. It is up to all those concerned in the promotion of the welfare of our people to take advantage of this propitious climate and to bend their energies towards ensuring better nutrition for our Nation.

ARYLSULPHATASES IN BRAIN

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Introduction

The sulphate esters present in the cells are hydrolysed by a group of enzymes, the sulphatases according to the general reaction.

$$R.O.SO_3^- + H_2O \longrightarrow R.OH + H^+ + SO_4^{2-}$$

The most thoroughly investigated group of sulphatases is the arylsulphatases. They catalyse the hydrolysis of arylsulphates or the sulphate mono esters of phenolic hydroxyl groups. They are widespread in their distribution and have been found in all animal species studied, in several microorganisms, higher plants and in molluses. Two groups of arylsulphatases type I and type II have been distinguished, by their substrate specificity, response to inhibitors and subcellular localization in ox, rat and human livers (1-3).

The type I arylsulphatases are more active towards simple substrates such as p-nitrophenyl sulphate and p-acetylphenyl sulphate. They are much less active towards p-nitrocatechol sulphate. They are generally inhibited by cyanide and sulphite but hardly affected by chloride, fluoride, phosphate and sulphate ions. The type I arylsulphatases of mammalian liver, the arylsulphatases C, differ from the corresponding enzymes of microorganisms in their extreme insolubility (4-6). They are localized in the microsomes and very difficult to solubilise. The only soluble arylsulphatase C which has been obtained was prepared from rat liver microsomes by treatment with crude pancreatic enzymes in the presence of a non-ionic detergent (4).

Type II aryl sulphatases have been detected in plants, animal tissues and microorganisms. The most studied examples are the arylsulphatases A and B of mammalian livers (5, 6). The type II arylsulphatases are characterised by their high activity towards p-nitrocatechol sulphate and much less activity towards simple substrates. These enzymes are strongly inhibited by sulphite, phosphate, sulphate, and fluoride ions but not by cyanide ions. The extraction and purification of arylsulphatases A and B from ox and human brain have been recently described by various workers (7-9).

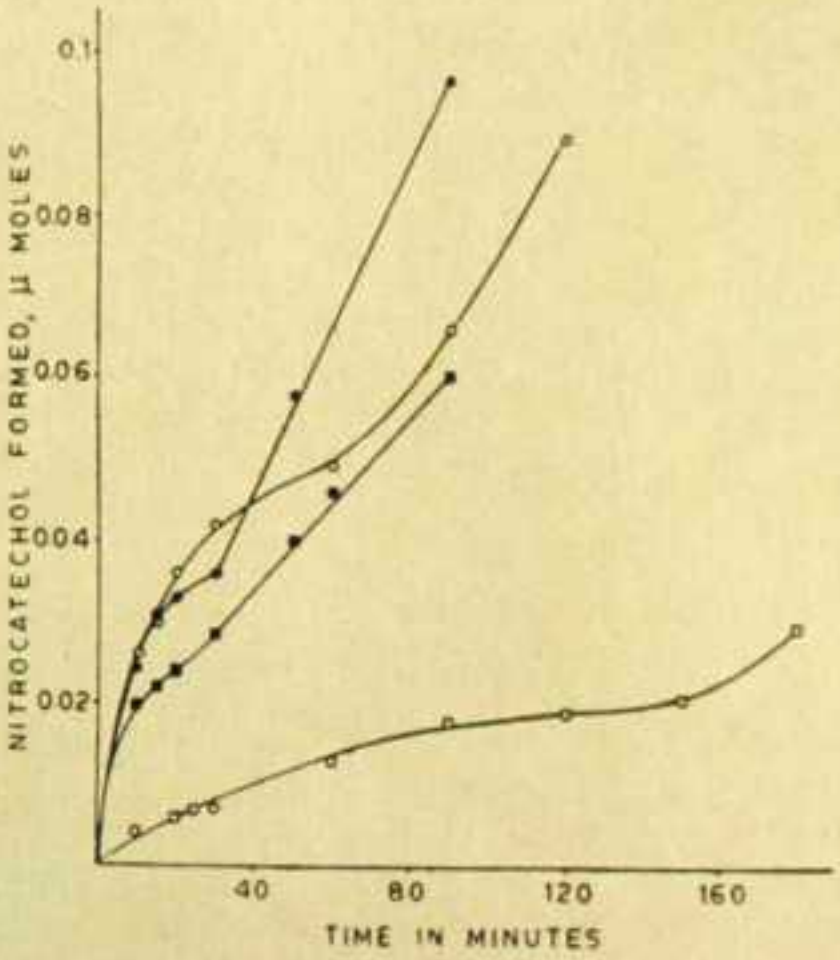
0.1
0.08
0.06
0.04
0.02
NITROCATECHOL FORMED, μ MOLES
40
80
120
160
TIME IN MINUTES

Fig. 1

Assay of arylsulphatases A and B :

There have been difficulties in assaying arylsulphatases A and B when they are present together, as in human urine. A solution to this problem has been worked out by Baum, Dodgson and Spencer (10) by utilising a differential inhibition with chloride ions and other small differences in properties. The determination of arylsulphatase A in the presence of B is based upon the following principles :

1. 'Normal Kinetics' are exhibited by arylsulphatase A at low substrate concentration in the presence of 2.5×10^{-4} M-sodium pyrophosphate.
2. Considerable inhibition of arylsulphatase B occurs under these conditions.
3. Arylsulphatase B is specifically inhibited by chloride ions when p-nitrocatechol sulphate is used as substrate, whereas arylsulphatase A is not.

The determination of arylsulphatase B in the presence of A is based on the fact that arylsulphatase A exhibits only a little residual activity after the first 20 minutes of the reaction when incubated at high substrate concentration in the presence of barium ions and in the absence of interfering ions. Moreover, this residual activity is linear and proportional to the intercept obtained by extrapolating this line back to zero time. Barium ions do not affect the activity of arylsulphatase B. According to Dodgson and his co-workers (11) this assay procedure may not be applied to rat liver arylsulphatases A and B because the differences in the kinetic properties of these enzymes are not sufficiently great to enable the independent assay of one enzyme in the presence of other.

Distribution of arylsulphatases :

Many studies of the distribution pattern of these enzymes have been made, both by conventional and by histochemical techniques. However, many of the results are difficult, if not impossible, to interpret adequately because of the failure of investigators to appreciate the complexity of the enzymes. The most reliable information on the distribution of arylsulphatases in mammalian tissues is that of Dodgson and his group for the rat (12) and the human (13). They have shown that, in general, liver is the organ richest in arylsulphatases and that considerable amounts also occur in the kidney, pancreas and adrenal. Arylsulphatase activity in nervous tissue was first reported by Neuberg and Simon (14) in rabbit brain. Arylsulphatases occur in human urine and serum also (15, 16). The soluble arylsulphatases have also been found in epiphyseal, articular and rib cartilages and in metaphysis and bone marrow of rat (17). In addition arylsulphatase activity has been detected in some plants (18) fungi (19) and bacteria (20).

Arylsulphatases have been detected in all animal species (21, 22). The quantitative date are available only for liver. Here also the separate determination of arylsulphatase A and B have not been carried out, and hence the proportion of these enzymes are only approximately known. Roy (21) has studied the proportions of arylsulphatases A and B by separating them by paper electrophoresis and according to him the enzymes corresponding to arylsulphatases A and B are detected in all the species. Recent comparative studies in this laboratory (22) on the activity of arylsulphatases A and B in brains of different animal species show that the proportions of these enzymes vary from one species to another (Table 1). In a lower vertebrate like frog the ratio of arylsulphatases A : B is 1 : 1. In birds, like pigeon and chicken arylsulphatase A accounts for the greater part of the total arylsulphatase activity. In fact in chicken the activity of arylsulphatase A is exceptionally high compared to B. In mammals like rabbit and sheep arylsulphatase A is high while in mammals like rat, monkey and man, arylsulphatase B predominates.

Balasubramanian and Bachhawat (23) studied the regional distribution of arylsulphatase activity in sheep brain and they found that the activity of arylsulphatases was high in those regions which were rich in white matter. Recently Farooqui and Bachhawat (22) studied the regional distribution of arylsulphatases A and B in monkey brain (Table 2). The data on regional distribution suggest that arylsulphatase A is always high in white matter not only in cerebrum but also in regions like medulla and corpus callosum. The activities of arylsulphatase B are high in grey matter compared to white matter in most of the regions.

Roy (21) studied the intracellular distribution of arylsulphatase in liver and according to him arylsulphatases A and B occur in lysosomes while arylsulphatase C is found exclusively in microsomes. These findings have been recently confirmed by cyto-chemical staining method (24). Clendenon and Allen (25) studied the subcellular distribution of arylsulphatases in rat brain and they found that the localization of these enzymes was the same as in the liver. Roy (26) has reported that arylsulphatase B of ox liver is localized in the framework of the lysosomes while arylsulphatase A in the sap.

Properties of arylsulphatases :

In general arylsulphatase A is a very acidic protein with an isoelectric point at pH 3.4. The enzyme exists as a monomer (M.Wt 107000) at pH 7 but as the pH is lowered towards the acidic side the monomer shows an increasing tendency to associate to produce a tetramer of molecular weight 411,000. This enzyme has high affinity for p-nitrocatechol sulphate (27). The arylsulphatase

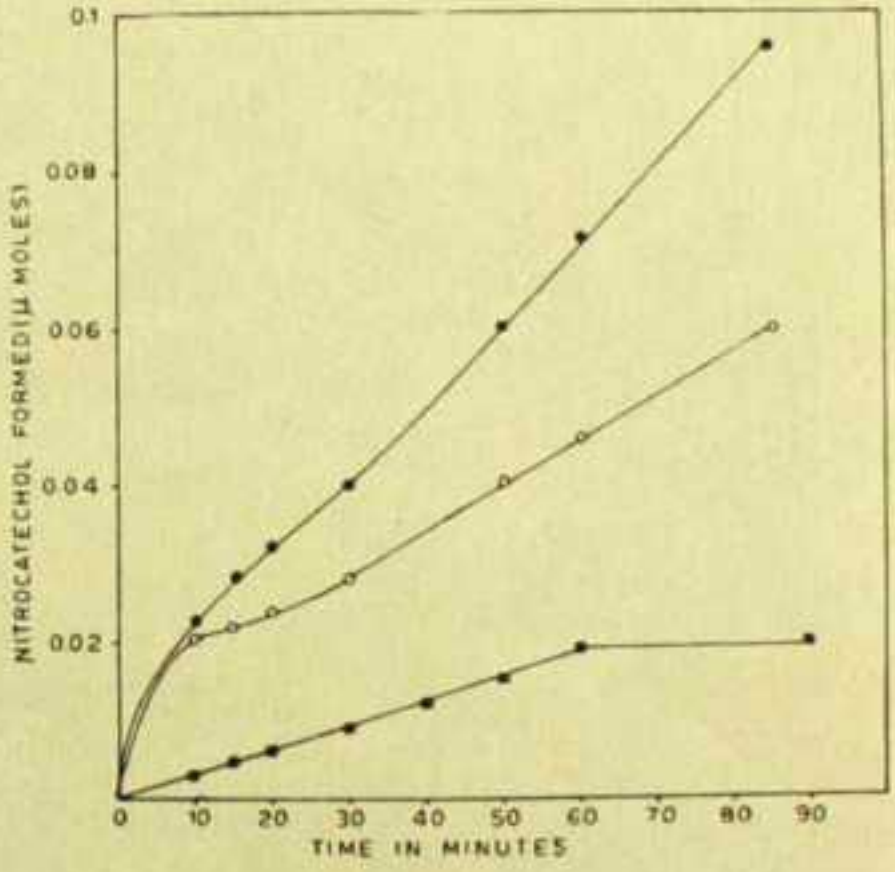
0.1
0.08
0.06
0.04
0.02
NITROCATECHOL FORMED(μ MOLES)
0
10
20
30
40
50
60
70
80
90
TIME IN MINUTES

Fig. 2

B is a slightly basic protein and has low affinity for p-nitrocatechol sulphate. More recently studies on arylsulphatase B from ox liver (28), ox brain (8) and rabbit cornea (29) have revealed that the enzyme can be resolved into further fractions by chromatographic or electrophoretic techniques. Allen and Roy (28) have purified and separated two of these forms (Bα and Bβ) from ox liver. Both have molecular weights of about 25,000 and are kinetically indistinguishable. Under certain conditions of low ionic strength, aggregates of these enzymes can occur which yield mixtures of polymers with molecular weights of upto 300,000. Electrostatic interaction is probably the main factor involved in this aggregation process. Comparison of some properties of arylsulphatases A and B is shown in Table 3.

The kinetics of arylsulphatase A are extremely complex, because the reaction velocity shows abnormal relationship with the enzyme concentration and time of incubation (30). The anomalous reaction kinetics of arylsulphatase A is manifested as a time-dependent loss of hydrolytic rate during incubation with p-nitrocatechol sulphate, followed by a partial recovery of the initial rate. Baum and Dodgson (30) have proposed " during the interaction of enzyme and substrate a new site capable of binding with substrate as well as the reaction products (p-nitrocatechol and sulphate) and certain other inhibitory compounds is slowly exposed to the enzyme. When the substrate is bound to the new site of the modified enzyme, the latter is virtually inactive. On the other hand when the reaction products or other inhibitory compounds are bound to the new site, the modified enzyme is active, although the active centre may still be inhibited if these compounds are present in excess." This hypothesis is recently confirmed by Nicholls and Roy (31) who actually isolated the so-called " inactive modified enzyme " from incubation mixture. Apparently " inactive modified enzyme " is strongly activated by sulphate, but only slightly activated by p-nitrocatechol. Phosphate and pyrophosphate, the inhibitors of the native enzyme, also activate the " modified enzyme." The low activity of the modified enzyme was found due to a powerful substrate inhibition which is decreased in the presence of sulphate. Furthermore it was postulated that sulphate displaces the equilibrium in favour of the native enzyme primarily through the formation of a " native enzyme-substrate complex." The anomalous time-activity curves for arylsulphatase A of different animal species are shown in Figure 1. Arylsulphatase B from different animal species follows normal kinetics (Figure 2).

Recently, Farooqui and Bachhawat (32) have purified a unique arylsulphatase A from chicken brain. This enzyme resembled arylsulphatase A of other animal species in its kinetic properties such as K_m value, anomalous

time-activity relationship and inhibitory effect of phosphate, sulphite and sulphate ions. However, its electrophoretic mobility, behaviour under zinc acetate fractionation, stimulation by Ag^+ and inhibition by citrate ion were similar to arylsulphatase B of other animal species. A similar arylsulphatase A is also recently purified from kangaroo liver by Roy (33). This enzyme shows less pronounced anomalous kinetics, does not polymerize at low pH values, had a different isoelectric point and different K_i value for sulphate ions compared with ox liver arylsulphatase A.

The chicken brain arylsulphatase A catalyses the desulphation of both p-nitrocatechol sulphate and cerebroside 3-sulphate (Figure 3 and 4). The comparison of some kinetic parameters of this enzyme using cerebroside 3-sulphate and p-nitrocatechol sulphate is shown in the Table 4. The non-ionic detergents stimulate the enzyme activity towards both cerebroside 3-sulphate and p-nitrocatechol sulphate (34). Like pig kidney cerebroside sulphatase (35) this enzyme has same pH value 4.5 and almost same K_m value 0.12 mM. Further with cerebroside 3-sulphate as substrate the enzyme shows a linear increase in (^{35}S) sulphate formation upto 60 min. and then the curve plateau's off (Figure 5). This linear time activity relationship can be compared with that obtained by Harinath and Robins (9) for human brain arylsulphatase A using 4-methylumbelliferone sulphate as substrate. These differences in the kinetic properties of arylsulphatase A may be attributed to the nature of the substrate. It should be noted that the relative rate at which this enzyme cleaves cerebroside 3-sulphate is 1,600 times less than the rate at which it cleaves p-nitrocatechol sulphate. A similar observation has been made by Mehl and Jatzkewitz (35) in the case of cerebroside 3-sulphatase of pig kidney.

The studies by Koenig and his co-workers (36) have indicated that essentially all the enzyme proteins of rat kidney and liver lysosomes are glycoproteins and that at least some of these glycorpoteins contain sialic acid. Recently, Goldstone, Koneeny and Koenig (37) have claimed that approximately 40% of arylsulphatase A can be converted to arylsulphatase B by neuraninidase treatment and the incubation without neuraminidase produced a smaller conversion of the arylsulphatase A into arylsulphatase B. The arylsulphatase B produced by neuraminidase treatment showed increased binding affinity for p-nitrocatechol sulphate and reacted as arylsulphatase B on biochemical assay. But it is really surprising because arylsulphatase A has more affinity for p-nitrocatechol sulphate and arylsulphatase B has less affinity for this synthetic substrate. On the basis of the acidic and basic nature of lysosomal hydrolases the above authors have proposed a deficiency of the specific sialyl transferase which produces arylsulphatase A, deficient in the genetic disorder metachromatic

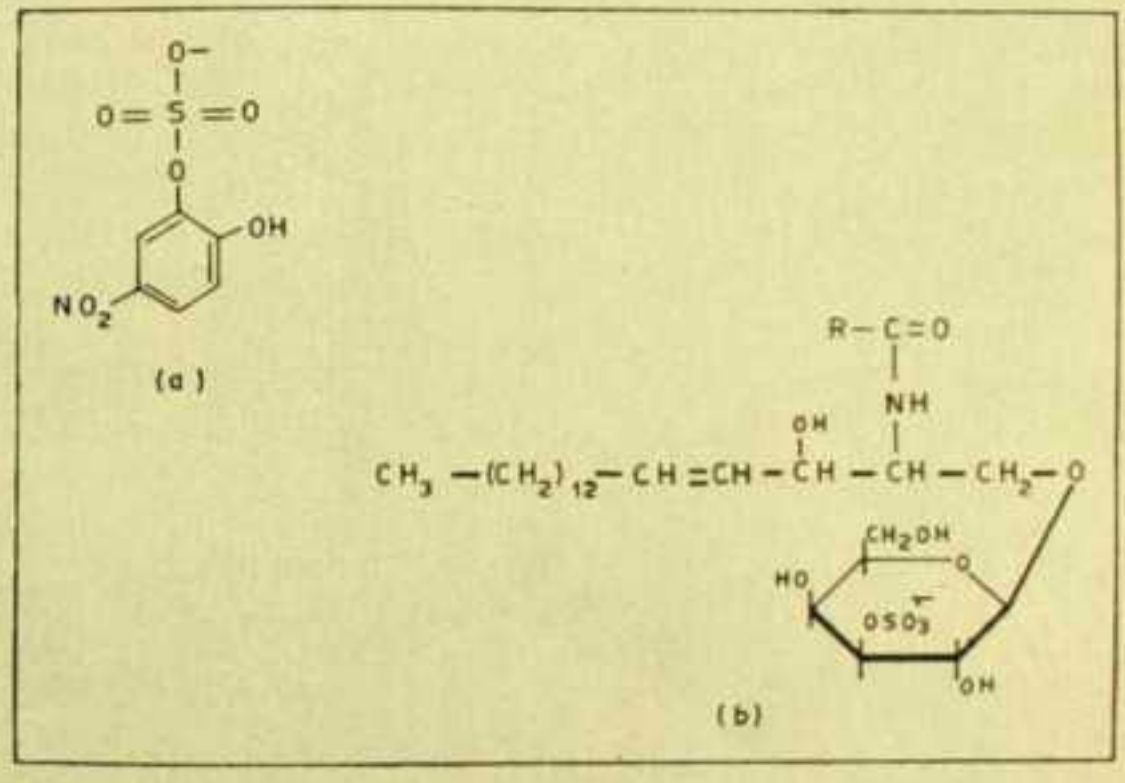

Fig. 3

leukodystrophy, from arylsulphatase B which is present in normal or elevated amounts in this disease.

Role of arylsulphatases :

Interest in arylsulphatases rose immensely after Austin *et al* (38, 39) using p-nitrocatechol sulphate as substrate had proved the deficiency of arylsulphatase A not only in tissues but also in the urine of patients affected by metachromatic leukodystrophy—a disease characterized by the absnormal accumulation of cerebroside 3-sulphate in brain, kidney and other visceral organs (Table 5 and Figure 6). Later, Mehl and Jatzkewitz (35) and Jatzkewitz and Mehl (40) explained the interrelationship of arylsulfatase A to cerebroside-3-sulphate by demonstrating that arylsulphatase A is a component of a cerebroside sulphatase —an enzyme splitting the cerebroside 3-sulphate. An especially interesting aspect in the reports of these investigators is the requirement for a heat stable factor which enhances the degradation of cerebroside 3-sulphate 7 to 10 fold by arylsulphatase A. Recent work from the laboratory of Jatzkewitz (41) has indicated that buffer concentration has a marked effect on enzyme activity when cerebroside 3-sulphate was used as substrate. There was an increase in enzyme activity upto a final concentration of 0.01M but as the buffer concentration was increased to 0.2 M there was almost complete inhibition of enzyme activity. Furthermore "complementary heat stable factor" is required only at high buffer concentration (0.2 M) for cerebroside 3-sulphate degradation.

Neuwelt *et al* (42) have reported that metachromatic leukodystrophy tissue extracts contain immuno-reactive proteins which precipitate anti-arylsulphatase A serum. These findings and the data of Porter *et al* (43) with fibroblasts provide evidence that arylsulphatase A proteins appear to be similar in that they all exhibit extreme lability under normal extraction ,procedures but they may differ among themselves in the affinity for cerebroside sulphate or in some other parameter which determines latency of clinical onset. The studies by Porter *et al* (43) and the wide range of reported latencies in clinical manifestations suggest that the metachromatic leukodystrophies result from a broad spectrum of cerebroside sulphatase deficiency ranging from virtual absence to an appreciable percentage of normal levels in those cases of late onset of symptoms.

The role of arylsulphatase B is quite obscure. However, a marked increase in arylsulphatase B activity was observed in Hurler's syndrome (44). Further it was found that although there was increase in heparan sulphate and dermatan sulphate fraction in Hurler's disease compared to normal, the total mucopolysaccharide content in Hurler's and normal brain was the same. Recently

Abraham *et al* (45) reported that there was a marked increase of arylsulphatase B in the biopsy samples of patients with Sanfilippo syndrome compared to arylsulphatase A and the ratio of arylsulphatase B to A was very high compared to that of normal brain (Table 6). Held and Buddecke (46) using (^{35}S) chondroitin 4-sulphate of bovine nasal septum, have purified chondroitin 4-sulphatase free from arylsulphatase and hyaluronidase from bovine arterial tissue and showed that this is a specific enzyme acting at polymer level. The enzyme has pH optimum at 4.4 in acetate buffer. The release of inorganic sulphate from unlabelled chondroitin 4-sulphate could be demonstrated turbidimetrically by converting it to barium sulphate, but this method did not show any action of the enzyme on chondroitin 6-sulphate, dermatan sulphate or keratan sulphate. The above authors also claimed that the addition of hyaluronidase did not stimulate the release of sulphate by this enzyme. Very recently Tudball and Davidson (47) have shown the existence of an enzyme related to that of bovine aorta and yet distinct in lysosomes of rat liver. Although partially purified, the enzyme was contaminated with β-glucuronidase, β-acetylglucosaminidase and arylsulphatase ; its substrate specificity was curious in that it was most active with high molecular weight oligosaccharides derived from (^{35}S) chondroitin 4-sulphate of chick embryo by the action of hyaluronidase. The enzyme had less action on low molecular weight oligosaccharides and none on the parent polysaccharide ; the removal of the nonreducing glucuronosyl residue of the oligosaccharide substrate was apparently essential for enzymic activity. Lloyd (48) and his associates have shown that mammalian sulphamidase liberates inorganic sulphate from heparan sulphate by the cleavage of sulphamido groupings. Recently, a sulphamidase has been purified from mammalian sources by Dietrich (49). The relationship of arylsulphatase B to these enzymes acting at oligosaccharide and polysaccharide levels is not known.

Recently Murphy *et al* (50) have described that the tissues of a metachromatic leukodystrophy patient showed not only the deficiency of arylsulphatase A but also arylsulphatase B, C and steroid sulphatase. This patient showed the accumulation of cerebroside 3-sulphate, cholesterol sulphate and glycosaminoglycans. A similar varient of metachromatic leukodystrophy with elevated levels of cerebroside 3-sulphate, ganglioside and glycosaminoglycans was also described by Austin *et al* (51). This patient showed the deficiency of arylsulphatase A, B and C. The contistent pattern of deficient sulfatase activities in the tissues of two such unrelated patients, the normal activities of other acid hydrolases, and the elevated tissue levels of relevant substrates indicate that the multiple deficiencies in the patients studied by Austin and his collaborators (38, 39) and by Murphy *et al* (50) are not artifacts. Auerbach and DiGeorge

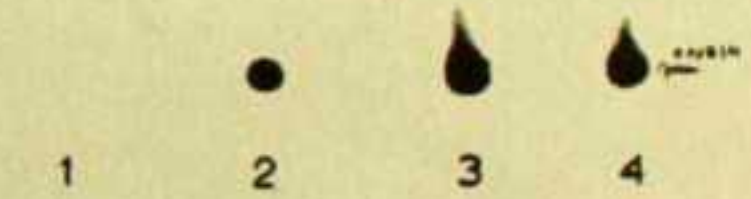

Fig. 4

(52) have presented a formulation by which multiple enzyme defects can be explained without violating the one gene—one enzyme concept. It is tempting to speculate that the multiple sulfatase defects present here may reflect a defect in a regulator gene, a phenomenon which is well established in bacterial systems, but not yet in man (53, 54). A defect in a regulator gene would imply that the areas controlling these enzymes are adjacent on a chromosome. Evaluation of these issues requires more precise information about the structure and function of the various sulphtases and, eventually the construction of an accurate genetic map. Murphy *et al* (50) suggested that the detailed study of patients with multiple enzyme defects may help to resolve these fundamental aspects of human genetics.

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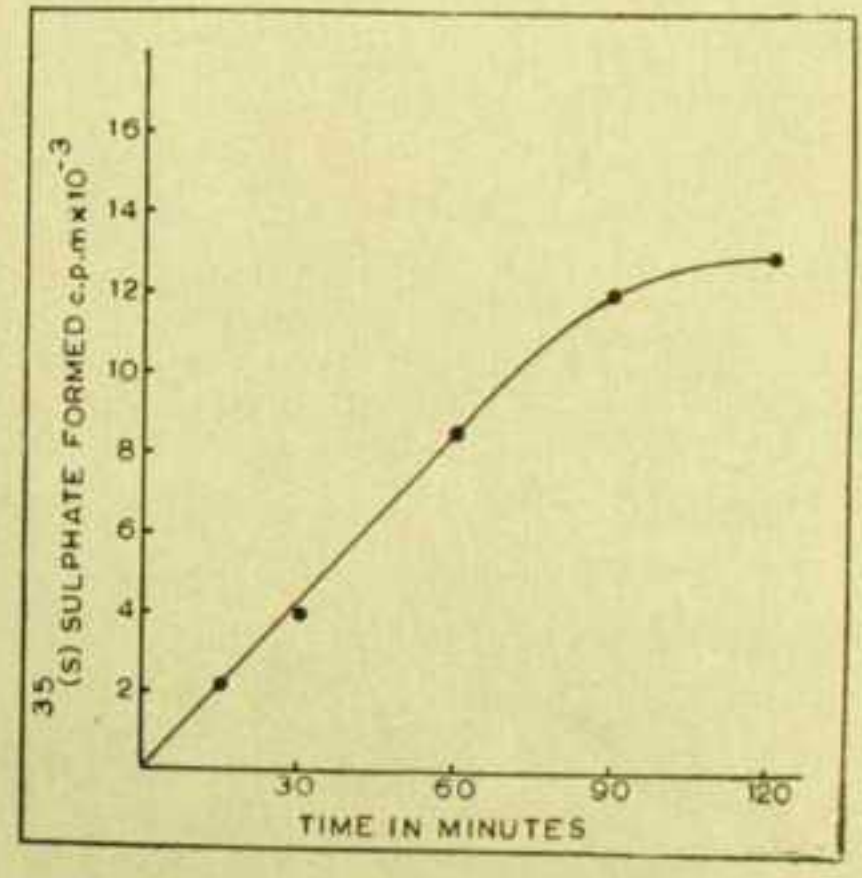
35 (S) SULPHATE FORMED c.p.m x 10^-3
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TIME IN MINUTES

Fig. 5

ACKNOWLEDGEMENT

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TABLE 1

Ratio of specific activities of Arylsulphatase A and B in brains of various animal species

Species	Specific activity A	Specific activity B	Ratio of specific activity A : B
Rat ..	0.28	0.58	1 : 2
Man (adult) ..	0.1	0.16	1 : 1.6
Child (2 years) ..	0.06	0.07	1 : 1
Hyrler's syndrome patient (7 years) ..	0.03	0.17	1 : 5.6
Sanfilippo syndrome patient (8 years) ..	0.02	0.09	1 : 4.5
Monkey ..	0.07	0.12	1 : 1.7
Sheep ..	0.16	0.06	2.6 : 1
Rabbit ..	0.08	0.06	1.3 : 1
Pigeon ..	0.07	0.03	2.3 : 1
Chicken ..	0.1	0.003	33.3 : 1
Frog ..	0.02	0.02	1 : 1

Specific activity is expressed in μ moles of nitrocatechol formed/mg protein/hr.

TABLE 2

Ratio of specific activities of Arylsulphatase A and B in different regions on monkey brain

Regions	Specific activity Arylsulphatase A	Arylsulphatase B	Ratio of specific activities A : B
Cerebrum :			
Frontal grey ..	0.04	0.07	1 : 1.75
Frontal white ..	0.10	0.09	1.1 : 1
Parietal grey ..	0.05	0.11	1 : 2.2
Parietal white ..	0.05	0.12	1 : 2.4
Occipital grey ..	0.08	0.15	1 : 1.8
Occipital white ..	0.10	0.08	1.2 : 1
Cerebellum ..	0.04	0.06	1 : 1.5
Quadrigeminal bodies ..	0.07	0.16	1 : 2.2
Pons ..	0.10	0.14	1 : 1.4
Medulla ..	0.12	0.08	1.5 : 1
Corpus Callosum ..	0.09	0.06	1.5 : 1

Specific activity is expressed in μ moles of nitrocatechol formed/mg protein/hr.

TABLE 3

Differences in properties of Arylsulphatases A and B of ox liver

Properties	Arylsulphatase A	Arylsulphatase B	Reference No.
Electrophoretic mobility	Moves towards Anode	Moves towards cathods	57
Isoelectric point	3.4	8.2	27, 28
pH optimum	4.6-5.2	5.0-6.0	55
Km (mM)	0.81	α 1.86—0.08 β 1.9 —0.09	55, 28
Effect of sulphate	Inhibited competitively	Inhibited noncompetitively	58
Effect of silver nitrate	Marked inhibition	Slight stimulation	9
Effect of chloride	No effect	Inhibited	28
Molecular weight	107000	25000	28
Effect of lowering the pH	Forms Tetramer	Forms nonspecific aggregate	27, 28

TABLE 4

Some Kinetic Parameters for the chicken brain Arylsulphatase A

Parameter	Cerebroside 3-sulphate	p-Nitrocatechol sulphate
pH optimum	4.5	5.5
Km (mM)	0.12	0.81
V_{max} (nmol/min/mg)	1.5	2500

TABLE 5

Specific activity of Arylsulphatase in different organs of patients with disorders of glycolipid metabolism

Sample and diagnosis	Arylsulphatase unit/mg protein)			
	Brain	Liver	Kidney	Urine
Normal	88.6	55.0	71.3	52.2
Metachromatic leukodystrophy	1.1	10.4	3.9	(0.3-4.3)
Normal	33.5	29.7	56.4	
Globoid leukodystrophy	31.9	53.8	74.0	
Gargoylism	ND	89.0	89.6	
Metachromatic leukodystrophy	3.1	6.9	ND	
Normal	52.1	ND	ND	

ND—Not done.
One unit of enzyme was defined as 1×10^{-3} μ mole of p-nitrocatechol liberated in 30 min.

TABLE 6

Arylsulphatase A and B activities in Hurler and Sanfilippo syndrome

Biopsy	Age	Arylsulphatase A	Arylsulphatase B
Normal	16	26.1	56.8
Normal	7	83.0	213.0
Normal	4	14.9	46.9
Hurler	4	46.0	400.0
Sanfilippo	7	72.0	400.0
Sanfilippo	11	55.0	522.0

Specific activity is expressed in μ moles of p-nitrocatechol formed/mg protein/hr.

LEGENDS FOR FIGURES

Fig. 1. Time-activity curves of rat, monkey, sheep and chicken brain arylsulphatase A.
Rat ( ); Monkey ( ); Sheep ( ); and Chicken ( ).
Fig. 2. Time activity curves of arylsulphatase B of various animal species.
Rat ( ); Monkey ( ); and Sheep ( ).
Fig. 3. Structure of (*a*) p-nitrocatechol sulphate, and (*b*) cerebroside 3-sulphate, in which R—the alkyl chain of a fatty acyl group.
Fig. 4. Radioautogram showing the electrophoretic separation of (^{35}S) sulphate from (^{35}S) cerebroside 3-sulphate.
1. Standard (^{35}S) sulphate ; 2. Reaction mixture ; 3. Control reaction mixture, and 4. (^{35}S) Cerebroside 3-sulphate.
Fig. 5. The time-course of (^{35}S) sulphate formation from (^{35}S) cerebroside 3-sulphate by chicken brain arylsulphatase A.
Fig. 6. Thin layer chromatogram showing the high concentration of cerebroside 3-sulphate in metachromatic leucodystrophic patient.
C—Standard cerebroside.
S—Standard sulphatide.
M—Metachromatic leucodystrophic patient.
N—Normal.

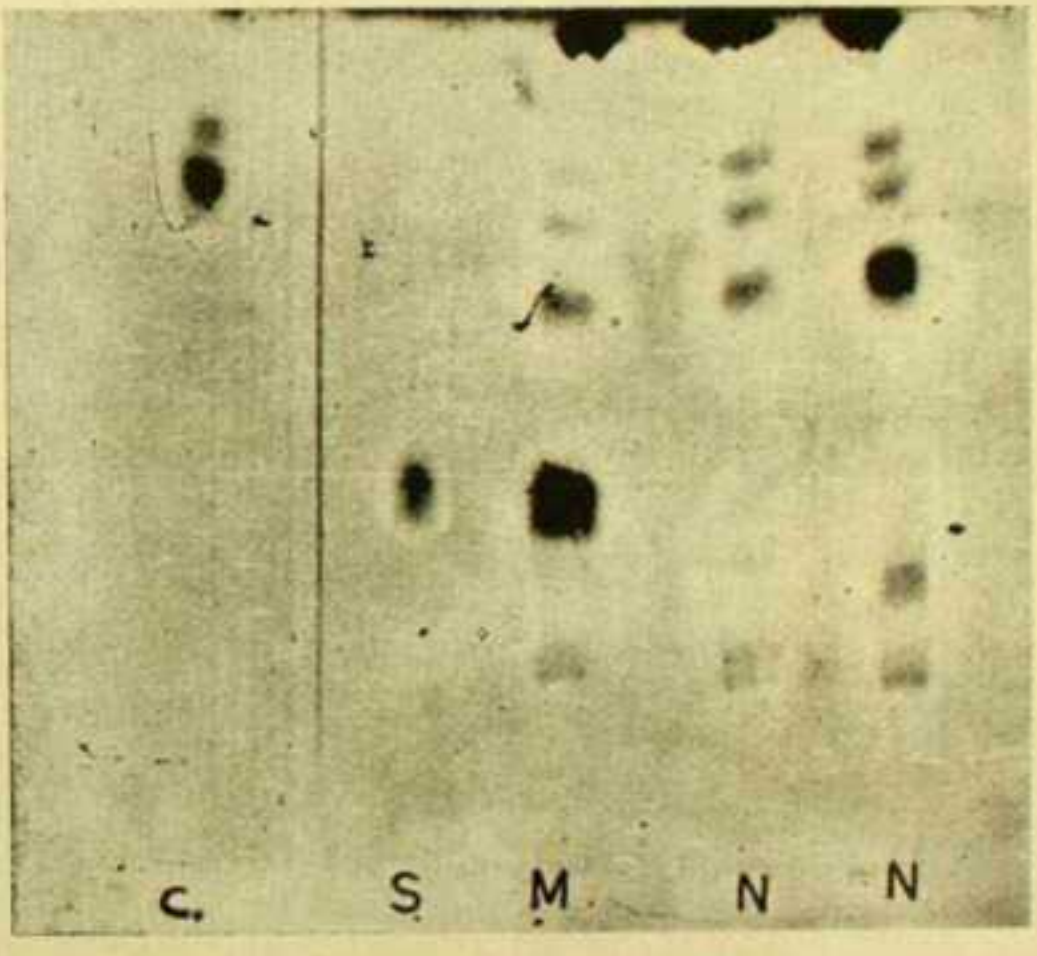

Fig. 6

ASCORBIC ACID METABOLISM DURING PREGNANCY AND LACTATION

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It is well-known that reproductive performance imposes a physiological and nutritional stress on the maternal organism partly because of the physiological changes resulting from the same but mainly because of the parasitic nature of the growing fetus and nursing infant. When the maternal diet is already poor it is likely to become more so with the advent of pregnancy and lactation with the possibility of serious consequences for either the mother or the infant or both. The problem may be acute in the case of critical nutrients such as iron, iodine, and thiamine. For instance infantile beriberi due to maternal deficiency of thiamine and a high mortality due to the same are common in communities consuming polished rice. Cretinism and deaf-mutism are prevalent in goitre areas. Anemia is more common in the children of iron deficient mothers (Bhatt *et al*, 1969). It is also known that the incidence of low birth weight babies, miscarriages, still-births and neonatal deaths is higer in communities with a low plane of nutrition (Rajalakshmi, 1971).

Ordinary diets in this country including those consumed by pregnant and lactating women are deficient in most nutrients and the deficiencies are critical in the case of calories, protein, calcium, vitamin A and riboflavine. Although the diets are apparently adequate in iron, iron deficiency anemia is also common. It is not surprising therefore that clinical, biochemical and radiological symptoms associated with deficiencies of these nutrients have been frequently reported in pregnancy. They include anemia, low levels of serum albumin, xerophthalmia and night blindness, osteomalacia, cheilosis and angular stomatitis (Rajalakshmi, 1971).

One would expect the above list to include clinical or subclinical scurvy as ordinary diets consumed by the poor contain 10-15 mg of vitamin C or less and the fetus at full term contains a store of 700-1000 mg. of vitamin C. Milk secretion during lactation involves an output of 20-30 mg of the vitamin and to this amount must be added urinary output. Yet reports of scurvy either in the breastfed infant or the pregnant and nursing mother are conspicuous by their absence.

We were alerted to this anomaly more than a decade ago when during the course of investigations on the relation between the composition of diet and

milk with regard to different nutrients including vitamins, we found the output of vitamin C in milk to be more than intake from the diet (Rajalakshmi, Deodhar, and Ramakrishnan, 1965). Yet these women showed respectable levels of vitamin C in blood. Naturally, we were baffled. Several questions seemed to rise including those listed below :

1. Are the estimates of dietary intakes under-estimations ? Do the women sporadically take rich sources of the vitamin such as amla and other common berries and get their tissues replenished ?
2. Are the figures of milk yield reliable ? Is the concentration of the vitamin in milk maintained throughout lactation ?
3. Do the levels of the vitamin in urine and blood decrease with the progress of pregnancy and lactation ?
4. If not, could tissue depletion during reproduction and repletion during the intervals in between account for their normal vitamin C status ?
5. Is it possible that ascorbic acid is synthesized in the maternal tissues either normally or adaptively during pregnancy and/or lactation ? If so, what is the site and mechanism of such synthesis ?

To get reliable estimates of dietary intakes, the foods consumed in typical families were collected at meal-time in air-tight containers brought under ice to the laboratory and analysed immediately. This was done for 23 families for a period of seven consecutive days. Tables were prepared from the data obtained on the ascorbic acid content of fruits and vegetables as consumed (Rajalakshmi and Kothari, 1964).

In the studies on pregnant and lactating women, 42 subjects in the poor group and 18 subjects in the upper class were studied from early pregnancy till six months or more of lactation. During this period, daily records were kept of the intake of all possible sources of ascorbic acid including fruits, vegetables, sprouted legumes etc. Aliquot protions of the foods consumed by the subjects were collected for two consecutive days each month and analysed and the analysed values found to agree well with estimates from previously prepared tables and any minor differences disappeared in the average values (Rajalakshmi and Ramakrishnan, 1969).

These studies confirmed our previous impressions that the ascorbic acid intake of poor women is no more than 15-20 mg. The diet consumed by these families are rather monotonous consisting of roti and tea in the morning, and either roti and dal or vegetable or khichri and kadhi for the major meals of the day. Vegetables were usually consumed only once a day if at all and the average per capita consumption was about 1000 g. mostly in the form of onions, potatoes, brinjal and bottle gourd. Even the vegetables rich in vitamin C

such as cabbage and fenugreek leaves lost most of their vitamin by the time they were cooked, the technique of cooking cabbage being similar to that used by Bransby *et al* (1950) for providing a low vitamin C diet.

We also found that food fads and prejuidices operate against the consumption of seasonal berries and fruits rich in the vitamin. Amla, chani bor, phalsa, wood apple etc. are avoided by pregnant and nursing mothers although children are allowed to eat them occasionally. The desi mangoes consumed occasionally by them when in season do not contain much of the vitamin.

It must also be pointed out that our figures for vegetable and fruit consumption agree with all India figures for production and availability and with figures given in various diet surveys including the 843 surveys compiled by Mitra (1953). In other areas of the country such as Tamil Nadu or Kerala, where the poor often have a meal of rice and fish curry, vegetable consumption may be even less.

Thus both underestimation of dietary intake and the sporadic consumption of fruits and vegtables rich in the vitamin seemed to be ruled out.

Having checked our values for dietary intake, the next question that arises is, do the women in fact lose as much vitamin C as was suspected in previous studies ? To ascertain this, milk yield was determined by weighing the child before and after each feed for two consecutive days each month. The weighing was done by a field worker who practically stayed with the family during the period. In any event, any error is likely to be in the direction of underestimation rather than overestimation. Further, our milk yield figures of about 700 ml were consistent with those reported from Hyderabad (Venkatachalam, Susheela and Rau, 1967) and with estimated calories available per kg of body weight (70-90) in entirely breastfed infants and the weight gains shown by them (about 20 g per day).

The next question that arises is regarding the reliability of our estimates regarding the concentration of the vitamin in milk. In our studies the same was found to be 3 mg. per 100 ml in poor women and some-what more in upper class women (4 mg). These values are well in agreement with those obtained in previous studies in this laboratory (Deodhar *et al*, 1963) and elsewhere (Kon and Mawson, 1950 ; Belvady and Gopalan, 1959) and with the value reported in the food tables of the ICMR (Aykroyd, Gopalan and Balasubramanian, 1966).

In those studies, fore-milk samples of milk were collected between two feeds. Several studies have shown that the ascorbic acid concentration of fore-milk and after-milk samples does not vary (Kon and Mawson, 1950) nor does it vary with the interval after feed. The milk was collected and analysed

for two consecutive days such month so that seasonal variations are not likely to influence the results. In previous studies in this laboratory the vitamin C concentration of milk was not found to change appreciably in a group of women studied over a period of 10 months unless their diet was supplemented with vitamin C (Deodhar, Rajalakshmi and Ramakrishnan, 1963).

The values for urinary output averaged 6-7 mg a figure consistent with other reports on lactating women (Bagchi, 1958 ; Rajalakshmi and Kothari, 1964), pregnant women (Bagchi, 1958), school children (Sail, 1970), pre-school children (Ramachandran, 1968) and young adults (Ranganathan and Sankaran, 1937).

Thus our studies on poor women showed an average dietary intake of 15-20 mg of vitamin C as against an output of 25-30 mg in milk and urine and an average negative balance of about 10 mg. Even the upper class subjects were only in neutral balance. In other words they had no vitamin left for their own metabolism. It must also be pointed out that in several cases, the discrepancy between output and input exceeded these averages, and 12 out of 42 subjects were losing more than 10 mg per day. Even assuming an experimental error of this magnitude the data need explanation.

In the classical studies conducted by Crandon, Lund and Dill (1940) and more recently by Hodges *et al* (1969) the biochemical and clinical symptoms of vitamin C deprivation showed the pattern shown below :

Course of changes found in experimental Vitamin C deprivation

	Days for disappearance of ascorbic acid
Urine	20-25
Plasma	40-45
Whole blood	80-90
Platelets	120-130*

*Clinical symptoms appeared 1-2 weeks after this.

These symptoms must be expected to occur earlier in lactating women as they were losing vitamin C from the body and therefore worse than subjects maintained on a scorbutic diet. However, thorough clinical examination by Dr. Bagchi at different stages of pregnancy and lactation showed no evidence of perifollicular hyperkeratosis or hemorrhage or hemorrhage of the interdental papillae or other symptoms associated with vitamin C deficiency.

The blood concentration of vitamin C was found to be of the order of 0.5 mg per 100 ml in non-pregnant and non-lactating women. This value was found to be higher in late pregnancy (0.65) and to fall to about 0.5 mg after partum and to 0.4 mg by the end of six months of lactation. Thus women at

the end of six months of lactation had respectable levels of vitamin C in blood whereas the same should have disappeared well before three months. Similar observations were made earlier by Bagchi (1958) and on serum by Rajalakshmi *et al* (1965). As a matter of fact, in the studies of Bagchi, the pregnancy values were much higher and no decrease whatever was noted during lactation. In this connection, studies in Hyderabad have shown a rise in milk ascorbic acid with the advent of pregnancy in nursing mothers. Some investigators have reported a fall in plasma ascorbic acid during pregnancy (*e.g.* Teel, Burke and Draper, 1938 ; Belvady and Gopalan 1959). The differences could be possibly due either to a difference in the distribution of ascorbic acid in the plasma and blood cells or due to racial differences which are found in the case of vitamin B_{12} (Low – Beer *et al*, 1968).

It must be pointed out that in most cases lactation continues till the child is 18-24 months old, the output being about 500 ml. or more between 6-12 months and 300 ml. or more between 12-16 months.

Thus, during each pregnancy and lactation, a poor women must be deemed to lose to the fetus 700-1000 mg. and through milk a total or more than 8000 mg. not to mention the urine loss of about 5000 mg. The hypothesis of replenishment of these losses during the inter-pregnancy interval does not derive much support either from the data on dietary intakes or when we consider that many of these women become pregnant while they are still nursing the previous child and continue to do so well into the next pregnancy. Further, blood ascorbic acid was not found to decrease with increasing parity. In this connection Stojanow (1958) found no differences in blood vitamin C between women with children and those without.

To ascertain the extent of tissue depletion, if any, women at term, after partum and at six months of lactation as well as non-pregnant and non-lactating women were given load tests of the vitamin (500 mg. per day for 8 days). Again, no differences were found between the different groups. Essentially similar results were obtained by Bagchi (1968) and in previous studies in this laboratory (Rajalakshmi *et al*, 1965).

It must also be pointed out that while not many investigators have set out to study ascorbic acid balance during lactation, a similar negative balance is to be inferred from several other studies. For instance, Pathak (1958) reports that lactation is successfully maintained by beggar women around Jaipur on a diet containing less than 1 mg. of ascorbic acid per day. Even at an estimated milk yield of about 400-500 ml with an ascorbic acid concentration of about 2-3 mg. per 100 ml, and indeed without any lactation performance at all, the

data would need explanation. The subjects in this study are reported to have shown no evidence of any deficiency symptoms.

Bharachna (1956) subjected entirely breast-fed infants of mothers subsisting on common diets in Bombay to a detailed examination for nutritional status with regard to ascorbic acid using X-ray of the long bones, load tests, plasma level of the vitamin, red blood cell count, hemoglobin and the capillary fragility test. Except for gross unsaturation which is found even in well-nourished upper class subjects with princely levels of the vitamin in the diet as well as in blood, no other scorbutic symptoms were found.

Ingalls, Draper and Teel (1938) found mothers subsisting no diets low in the vitamin (less than 20 mg.) to secrete liberal amounts of the same (20-30 mg.) in milk. They also found a higher concentration of the vitamin in the plasma of the infant than in that of the mother.

It must be mentioned in this context that in spite of serious shortages during the war, normal blood levels of vitamin C (0.36 to 0.5 mg. per 100 ml.) were maintained by nursing mothers in Europe (Touverud, Stearns and McCy, 1950).

Other similar studies have been cited by Bagchi (1958) in his report for which full bibliographical details are not available.

Even apart from specific studies, there is no serious controversy over the fact that common diets in this country provide no more than about 100 g of vegetables and 15-20 mg. of vitamin C and that poor women secrete about 700 ml. of milk containing 3 mg. % vitamin C for prolonged periods. There is also no serious controversy about the fact that scurvy is seldom found in breast fed infants or nursing mothers.

It must also be pointed out that even in Europe, whatever may be the position with regard to vitamin C intake to-day, at the turn of the century or even till well after World War I, most of the poor families in the urban areas of Europe and North America lived on a diet of bread and beans. The nursing mothers in these families cannot be expected to have derived much more vitamin C from their diets than Indian women do to-day. Even in a relatively recent study on nursing mothers (Bransby *et al*, 1950) a considerable proportion of the subjects were found to subsist on diets providing less than 25 mg. of vitamin C. These women might be expected to have been secreting at least this amount in milk. It is indeed remarkable that no a single case of scurvy in a nursing mother appears to have been reported in the entire medical and nutritional literature.

In summary, nursing mothers in many areas of the world constitute a group in whom the supply of vitamin C is precarious as compared to the requirements of lactation. At least a small proportion of this group can be expected to have

scurvy or subclinical vitamin C deficiency and yet this has not been found to be the case.

The colorimetric method of Roe and Kuether (1943) was used in these studies for the extimation of ascorbic acid. A question may be raised regarding the reliability of the method. This method measures both ascorbic acid and its derivative 2, 3-diketo-L-gulonic acid and cannot therefore result in an overestimation of the net amount of the vitamin lost in milk and urine. On the other hand, there could have been an overestimation of the physiologically potent amount derived from foods. Any error therefore would be in the direction of underestimating the negative balance.

The only hypothesis that would account for the above data is that of synthesis of ascorbic acid in the body either normally or adaptively during pregnancy and/or lactation. The former possibility has been indeed suggested by Davidson and Passmore (1966) as well as Mitchell (1964) and others (Baker, Bierman and Plough, 1960). However, this hypothesis is hard to reconcile with the incidence of scurvy in human populations and its predictable onset with vitamin C deprivation although it is possible that the amount synthesized is not sufficient to meet all the requirements. In this connection, Baker *et al* (1960) have found evidence of ascorbic acid synthesis in man from labeled D-glucuronolactone.

The possibility of placental synthesis of ascorbic acid has been suggested by Bagchi (1958) who found no differences in the placental concentration of the vitamin between those who were supplemented with ascorbic acid and those who were not. Histochemical studies carried out by the same author using acid silver nitrate showed the presence and uniform distribution of reduced ascorbic acid. It is known that the placenta is not only a transmitting organ but also a highly active metabolic site capable of synthesizing highly complex compounds such as hormones and enzymes. According to Bagchi, the high concentration of ascorbic acid in the placenta cannot be due to storage as no such function has been attributed to it except for some reserves of glycogen and fat. There is not a similar high concentration of other nutrients which are transmitted generously to the fetus (*e.g.* calcium and iron). Nor is its structure suitable for this function.

In studies carried out in this laboratory, human placenta was found to contain the intermediates and enzymes of glucose metabolism. The former included glucuronic acid. Its oxygen uptake *in vitro* was quite high (170-190 ml. per g. per hour). Another interesting observation was that the distribution of ascorbic acid, dehydro-ascorbic acid and diketogulonic acid in the

placenta was very similar to that in the mature lemon which is known to synthesize ascorbic acid (Rajalakshmi and Rakamrishnan, 1969).

Investigations were also attempted on the enzymic synthesis of ascorbic acid in human placenta with the procedure used by Chatterjee *et al* (1957) with liver tissue. In the original studies only 27 out of 40 samples were found to show activity (Rajalakshmi *et al*, 1957). In subsequent studies it was found in all the samples. It is now felt that in the original series some of the samples might have lost their activity before they were assayed on the basis of the observation that this happened within 4 hours of storage in the deep-freeze in samples showing activity.

However, these studies suffer from the limitation that the product formed was determined by the dye-titration method which is not specific for ascorbic acid. The product formed was sought to be identified in selected samples by paper chromatography and the experimental showed a bigger spot than the blank.

Further studies using isotope techniques are needed to ascertain ascorbic acid synthesis in the placenta. We are encouraged to hear reports of similar findings by Walker in South Africa (Sir David Cuthberton, personal communication).

Even if we accept tentatively the hypothesis of placental synthesis of ascorbic acid, the difficulties are not quite solved. The question arises as to why in the face of such synthesis a greater spill over of ascorbic acid in urine does not occur during pregnancy. It is possible, however, that the vitamin, if synthesized, is stored in the uterine mucosa rather than allowed to spill over into the blood stream. Further, urinary excretion of the vitamin did not differ between poor and upper class women although their blood levels of the vitamin were different.

A question also arises as to the differences found between poor and upper class women with regard to milk, blood, and placental concentrations of ascorbic acid. In the event of placental synthesis, it is possible that amount synthesis, it is possible that amount synthesized varies with the nutritional status of the subject with regard to protein, vitamins etc. These factors have been found to influence liver synthesis of ascorbic acid in rats (Terroine, 1960 ; Rajalakshmi *et al*, 1967).

The lack of spill over in urine is also accounted for by the relatively low enzyme activity in the placenta as compared to the lemon fruit (Parekh, Sakariah and Shah, 1970) or rat liver. The activity was about 20% of that in the lemon fruit and 10% of that in rat liver. The weight of placenta is only 1% of body weight, whereas rat liver forms about 3-5% of body weight. If the results

present genuine synthesis of ascorbic acid in placenta, the rate of such synthesis in relation to body weight would be only 2% of the rate in rat liver on the basis of enzyme activity. In this connection turnover rates for ascorbic acid are found to be much slower in man (1 mg. kg. day) than in rats (26 mg. kg. day) (Hellman and Burns, 1958). It must also be pointed out that the metabolic activity of the placenta is considerably reduced att erm. If this indicates a relatively low rate of synthesis, it would account for the absence of dramatic effects on blood and urinary excretion. In any case it is possible that most of the vitamin synthesized is transferred to the uterine mucosa.

In conclusion, lactating women belonging to the low income group were found to be in prolonged negative balance with regard to vitamin C and yet had levels of blood vitamin C which corresponded to much higher intakes of the vitamin in the literature (Woodruff, 1964). Preliminary studies suggest the placental synthesis of ascorbic acid but this needs to be confirmed by more precise techniques. The inference of negative balance is based on data obtained on dietary intake, milk secretion and urinary excretion of the vitamin in nursing mothers. The data on the latter are consistent with a large body of information available on each aspect, and with similar data obtained by Dr. Bagchi in Calcutta.

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EFFECTS OF NUTRITIONAL DEFICIENCIES ON THE METABOLIC ACTIVITY OF THE BRAIN*

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When we started our investigations on the relation between nutrition and brain function, it was necessary to justify the conduct of such investigations to sceptics. Even the NRC-NAS report (1959) on protein listed the effects of protein deficiency on the CNS as nil. The past decade has witnessed the widespread acceptance of the possibility that brain development and function, specially in the young child or animal, may be affected by nutritional deficiency although not enough work has been done to justify generalisations about the type, duration and severity of deficiency that affects the CNS adversely, the parameters affected, the age or developmental stage at which such effects are found etc.

Among the parameters investigated the protein, DNA, RNA and lipid contents of the brain seem to be the most popular choices as development and maturation are associated with changes in the amount and concentration of these substances. Very few studies have been carried out on metabolic aspects.

This may partly be due to the fact that, till recently, the brain has been looked upon as a more or less static orgain undergoing no chemical change after reaching full development in early infancy. Such an assumption is no longer warranted. A rapid turnover of brain proteins has been indicated by isotope studies (Gaitonde and Richter, 1956). The brain is one of the most active metabolic sites and manufactures several of its constituents such as glutamic acid, the entry of the same from the blood stream being restricted by blood-brain barrier (Waelsch, 1957 ; Richter, 1962). Cerebral metabolism is found to account for 20-25 per cent of basal metabolism in the adult (Kety, 1957) although the weight of the brain constitutes less than 3 per cent of body weight. Such an active metabolic state implies a continuous requirement for, and availability of, enzymes, cofactors, amino acids etc., and it is hard to believe that these would not be affected by protein deficiency.

The brain compares with plasma and liver in its concentration of essential aminoacids, but has a much higher concentration of non-essential amino acids (Waelsch, 1957). Glutamic acid, glutamine and Gamma aminobutyric acid (GABA) account for a substantial portion of the latter. Most of brain glutamic

acid is locally synthesized (Strecker, 1957) and the rate of incorporation of labelled glucose in brain aminoacids is high as compared to that in the liver (Gaitonde, Dahl and Elliott, 1965).

Another consideration is that GABA and some other amino acids are uniquely present in the brain. GABA is of particular interest as glutamic acid and GABA are believed to have respectively facilitatory and inhibitory actions on dendritic activity in the brain (Jasper, Khan and Elliott, 1965). Although the electrophysiological role of GABA is a complex and controversial one (Curtis, 1963), the relative concentrations of glutamic acid and GABA may conceivably determine the level of background electrical activity in the brain. Further, the glutamate-GABA system provides a metabolic shunt in the brain accounting probably for the capacity of brain tissue to utilize for respiration glutamic acid and GABA but not other amino acids (Weil-Malherbe, 1936).

Till recently, conduction along the nervous system was believed to be an all-or-none affair, the brain functioning more or less like a telephone exchange. We now know that, in addition to this mode of transmission along the axons of nerve cells, there is another mode of transmission across the dendrites (Hebb, 1960). The axon either responds fully, or not at all, to stimulation (all-or-none response), whereas the response of the dendrite varies with the strength of the impulse (graded response). The latter results in diffuse transmission which does not reflect the original nature of the nerve impulse, but is very necessary for maintaining background electrical activity in the brain. Dendritic activity is evident in deep sleep and is believed to influence the level of arousal (Magoun, 1962).

The learning process is believed to involve the repeated firing of groups of neurons or neuronal assemblies (Hebb, 1949). Such firing cannot be efficient if the background electrical activity is too low, in which case it may not take place, or too high in which case the seqential firing of particular groups of neurons may become disorganized. We may therefore presume that an optimum level of dentritic activity is crucial for efficient CNS function. This may involve the maintenance of stable levels of glutamic acid and GABA and a proper balance between the two (Hebb, 1970).

These considerations prompted us to investigate the effects of different deficiencies on enzymes of glutamate-GABA system in the brain namely, glutamate dehydrogenase (GDH) and decarboxylase (GAD) and GABA transaminase (GABA-T). Additional studies were made of NAD glycohydrolase, because of the involvement of NAD as a coenzyme for glutamate dehydrogenase reaction and in the formation of adenine ribose needed for nucleic acid synthesis during NAD breakdown. The deficiencies were those of protein,

calories and vitamin A. Studies were also made of the effects of a diet poor in protein quality and of glutamic acid supplementation.

Additional data were obtained on the differential effects of protein deficiency on the distribution of selected enzymes in different regions of the brain, *in vitro* oxygen consumption, and concentrations of protein, glutathione and ascorbic acid.

The differences in oxygen consumption found between animals fed low and high protein diets led to a study of some of the enzymes involved in glycolysis and respiration.

In all these studies, unless otherwise stated, weanling albino rats weighing 40-50 g at start were used. Except for studies on protein quality, standard semipurified diets using starch (sago flour), vitamin-free casein, groundnut oil, salt and vitamin mixtures were used.

Protein :

Protein deficiency was found to produce significant deficits in body weight, brain weight, brain glutamate dehydrogenase and decarboxylase but had no effect on the activity of GABA transaminase (Rajalakshmi, Pillai, and Ramakrishnan, 1969). The effect was not due to a decrease in food intake with the low protein diet as no deficit was found in an additional control group pair-fed with the low protein group (Rajalakshmi, Jacob and Ramakrishnan, unpublished).

The above changes would be consistent with other changes reported with protein deficiency in studies cited earlier such as disturbed EEG patterns and poor learning capacity in animals and children. Such changes if they occur would perhaps account for tremors observed during recovery from kwashiorkor (Kahn, 1954 ; Udani, 1960) as such recovery may be associated with changing levels of glutamic acid and GABA.

As ordinary diets in poor countries are deficient not only in quantity but also in quality, studies were made of the effects of improving protein quality on these brain enzymes. Supplementation of kodri (Rajalakshmi, Pillai and Ramakrishnan, 1969), a millet containing 8.3% protein and so deficient in lysine that it is only equivalent to a 4.5% casein diet was found to increase the levels of GDH and GAD. Similar results were obtained with addition of lysine rich supplements such as milk powder, bengalgram or other pulses.

Similar supplementation of maize, wheat and bajra did not have a clearcut or consistent effect presumably because of their higher protein content (11-13%) as compared to kodri. However, the weight gains produced by maize and bajra corresponded to those produced by casein diets containing 5-6% protein whereas the wheat diet corresponded to one cotaining 6-7% protein.

As the original experiments on protein content were done with 5% and 20% protein and an increase in brain enzymes was obtained by supplementation of a diet containing only 7.3% protein without increase in nitrogen content, a question arose as to the critical level of protein needed to prevent the effects of protein deficiency on these enzymes. In a subsequent series of investigations protein content was varied at 5, 6, 7, 8, 10, 15 and 20%. With a protein content of 8% or more in the diet no deficit in enzyme activity was found (Rajalakshmi, Parameswaran and Ramakrishnan, unpublished). With seven per cent these were only slight whereas definite deficits were obtained with six per cent protein or less. In the light of these observations, a clearcut deficit in GDH and GAD should have been expected with the maize and bajra diets. The absence of such deficits suggests that their higher protein content and perhaps their higher concentration of individual aminoacids such as glutamate may compensate for their poor protein quality.

Since the amount of protein needed for rehabilitation of protein deficient animals may be more than that needed for preventing the effects of deficiency, animals fed a 5% protein diet for 10 weeks were rehabilitated with 8, 10, 15 and 20% protein diets. Enzyme activities were restored to normal levels with 10% or more protein in the diet but restoration was only partial with 8% protein (in the form of casein).

The question arose from the above observations whether decreasing the protein content below 5% would affect the size of the deficits. Dietary protein content was varied at 0, 1, 2, 3, 4, 5 and 20 per cent (Rajalakshmi, Parameswaran and Ramakrishnan, unpublished). At levels below 4% there was definite weight loss. With 4% protein (and about the same percentage of protein calories) body weights were just maintained. This is consistent with the observation that about 4 per cent protein calories are adequate for maintenance but not for growth (Miller and Payne, 1961). Decreasing protein levels below 5% was not found to decrease further GAD activity.

A most surprising observation was made when the protein content of the diet was reduced to nil or negligible amounts (0.2 per cent in the starch source used). Although, as expected, the animals lost weight and died if not killed earlier, the decrease in glutamic dehydrogenase was reversed. These remarkable results were confirmed by repeated experimentation and await a satisfactory explanation. They certainly suggest that the effect of complete protein deprivation may not always be the same as those of a low protein diet. The results are however consistent with the fact that the animals in these groups were losing weight and tissue catabolism might be expected to result in the release of ammonia for the removal of which maintaining a normal level of

glutamate dehydrogenase may be important. In this connection, it has been reported that during complete protein deprivation brain glutamate increases (Lehr and Gayet, 1966).

Studies were also carried out to identify the duration of deficiency needed for the production of measurable deficits. Groups of animals were killed at 1, 2, 3, 4 and 5 weeks after the initiation of treatment. Clearcut deficits in glutamate decarboxylase were observed only with 5 weeks but glutamate dehydrogenase was found to be affected at 3 weeks (Rajalakshmi, Parameswaran and Ramakrishnan, unpublished).

The above experiments were carried out on weanling rats. Since it is commonly believed that the effects depend on the age at which deficiency is produced, the experiments were extended to rats at 12 and 18 weeks of age. When treatment was begun at 12 weeks of age the animals were still affected but the effects were found at 10 weeks but not at 5 weeks suggesting their decreased susceptibility to the effects of deficiency. With older animals (18 weeks at start) no effects were found even with 10 weeks of treatment suggesting that the brain in the older animals may be less susceptible to deficiency as might be expected. Further studies are needed to see if a more severe deprivation for a longer period would affect the older animal.

In this connection, in man, diseases such as pellagra caused by protein-vitamin deficiencies and associated with CNS symptoms do occur in the adult (West and Todd, 1961). Mental symptoms and disturbed EEG patterns are found in the adult with a variety of deficiencies including that of vitamin B_{12} (Wokes, Badenoch and Sinclair, 1955).

Glutamic acid :

Several beneficial effects have been claimed for glutamic acid supplementation on the psychological performance in children (Zimmerman, Burgemeister and Putnam, 1949) and experimental animals (Zimmerman and Ross, 1944).

Supplementation with glutamic acid was found to reverse the effects of a low protein diet and restore enzyme concentrations to normal levels (Rajalakshmi, Pillai and Ramakrishnan, 1969) whereas supplementation to a high protein diet had no effect (Rajalakshmi, Jacob and Ramakrishnan, unpublished). The observation regarding the beneficial effects in the former case needs explanation as this amino acid is not known to cross the blood brain barrier although it may do so at a very slow rate. Also, under certain conditions, the blood brain barrier against this amino acid is broken by means of physical or chemical methods such as local freezing or ethyl chloride treatment

(Purpura *et al*, 1958). It is also possible that the LP diet may bring about changes in the operation of the blood brain barrier. In this connection, some histological studies suggest that the integrity of cell membranes in nerve cells may be affected in dogs subjected to severe protein deficiency (Platt and Stewart, 1969). Some changes in membranal structure would be consistent with the observation that brain tissue slices of low protein animals use glutamate more efficiently for respiration than those from high protein animals although the reverse was observed with homogenates (Rajalakshmi, Thrivikraman and Ramakrishnan, 1971c). The lack of any effect of glutamic acid supplementation in the case of the HP group may be due to the fact that the HP diet contains liberal amounts of glutamic acid even without such addition.

It is interesting to note in this connection that cereals such as maize and wheat contain generous amounts of glutamic acid (Naik and Das, 1972). This might account for the absence of brain enzyme deficits when these grains are fed although their protein quality is poor.

Calorie :

In the studies cited eartier, animals fed the high protein diet in restricted amounts did not show any enzyme deficits suggesting that the effects of calorie deficiency may be different from those of protien deficiency. Several studies confirmed this (Rajalakshmi, Pillai, Parameswaran and Ramakrishnan, unpublished). However, calorie deficiency during the neonatal period had a transient effect which was found at 2 and 4 weeks but disappeared when the animals were fed *ad libitum* after weaning (Rajalakshmi, Parameswaran and Ramakrishnan, unpublished). In the post-weaning period restriction of food on a 20% protein diet to half or one-third that of controls (5% protein diet) fed *ad lib* was without effect but a combination of neonatal undernutrition and post-weaning food restriction to 1/3 of control values was found to decrease both GDH and GAD. It has been suggested that even when the diets are adequate with regard to protein, when they are fed in restricted quantities, protein deficiency may result as a result of the use of protein as fuel. In the studies just cited, however, the brain enzyme deficits found in rats fed a protein deficient diet were not found in those fed a high protein diet in restricted quantities although the body weights of the former (72 g) were more than those of the latter (60 g). The question arises whether this situation would hold when the diet contains less generous but satisfactory amounts of protein. Preliminary results suggest that brain enzyme deficits are found when an 8% protein is fed in restricted amounts although when fed *ad lib* this diet is sufficient to prevent deficits.

Vitamin A :

Studies were made of the effects of vitamin deprivation using low and high protein diets. A deficiency of vitamin A was found to decrease the activities of both GDH and GAD (Rajalakshmi, Jacob and Ramakrishnan, unpublished).

In these experiments the vitamin was entirely omitted from the diet. It remains to be investigated whether these effects are also found with diets containing deficient amounts of the vitamin. Critical levels of vitamins needed for preventing these deficits should be identified.

Maternal deficiency :

Maternal protein deficiency had relatively little effect on body composition, psychological performance or brain enzymes in animals fed a normal diet after weaning although weaning weights were markedly affected (Rajalakshmi, Khanum, Upadhyay and Ramakrishnan, unpublished). This is perhaps not surprising as maternal protein deficiency affects the amount and not the protein content of milk secreted. Some transient effects before weaning cannot, however, be ruled out as such effects were found with food restriction during the neonatal period brought about by increase in litter size.

NAD glycohydrolase :

Essentially similar results were obtained with NAD glycohydrolase (Rajalakshmi, Rao, Thrivikraman, Parameswaran and Ramakrishnan, unpublished).

Psychological performance :

Most of the conditions which influenced brain enzymes also affected psychological performance as judged by a variety of parameters such as performance on the Hebb-William Maze, water maze, visual discrimination and reversal learning, locomotion scores and certain tasks involving psychomotor coordination.

Regional differences :

As the brain is far from a homogeneous bowl of porridge, the studies on protein deficiency were extended to different regions. As expected different regions of the brain showed variation in chemical composition and metabolic activity (Rajalakshmi, Thrivikraman and Ramakrishnan, 1971a,b,c). But it was interesting to note that they also differed in their susceptibility to the effects of protein deficiency.

Oxygen consumption under different conditions :

The oxygen consumption of brain tissue slices was found to be influenced by protein deficiency. In many brain regions oxygen uptake was found to be less in the LP animals when glucose was used as substrate. This difference was decreased or reversed with the use of glutamate as substrate (Rajalakshmi, Thrivikraman and Ramakrishnan, 1971c).

Enzymes of carbohydrate metabolism :

The differences in glucose utilization suggested the possibility of differences in the activities of enzymes concerned with carbohydrate metabolism. Among the enzymes investigated, activities of phosphofructokinase, lactate dehydrogenase, isocitrate dehydrogenase (NADP linked) and malic enzyme were found to be decreased by protein deficiency whereas hexokinase, aldolase and malate dehydrogenase were not affected. Phosphofructokinase, isocitrate dehydrogenase and aldolase were also decreased by vitamin A deficiency. It is interesting to note that one of these (aldolase) was not affected by protein deficiency.

Lipid composition of the brain :

Brain lipids were not found to be affected by protein deficiency during the post-weaning period but undernutrition during the neonatal period was associated with a decrease in total lipids, cholesterol and phospholipid.

Conclusion :

In conclusion protein and vitamin A deficiencies during the immediate post-weaning period were found to produce deficits in brain GDH and GAD and NAD-glycohydrolase but none in GABA-T. Similar effects were not found with post-weaning calorie restriction but found during the neonatal as well as post-weaning periods. The deficits were evident with a protein content of 6-7% or less and with five weeks of treatment. When 12 week old animals were subjected to deficiency, a longer period of treatment was found to be necessary to induce these deficits. With 18 week old animals, no deficits were found even at 10 weeks of treatment. Glutamate was found to reverse the effects of deficiency.

An important observation that arose in several studies was the absence of a consistent correlation between brain weight and brain enzyme activities. The former was sometimes affected when the latter were not, as in the case of calorie deficiencies in the post-weaning period. On the other hand, the reverse was true in the case of glutamic acid supplementation and certain other conditions.

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THE PROTEIN NUTRITION PROBLEM

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INTRODUCTION

Protein nutrition in young children is the major nutrition problem of the World and major cause of ill health. Over 300 million children suffer grossly from retarded physical growth and development because of lack of right food and deficiency of protein. Protein deficiency is common in the developing countries but sub-clinical deficiency is more prevalent. According to FAO's estimate about 1500 million people do not get food of right quality of which shortage of protein is the most significant cause of malnutrition.

Although protein deficiency may predominate yet calorie deficiency along with protein deficiency known as Protein-Calorie Malnutrition (PCM) complicates the situation more gravely. This may occur at all ages but its impact is greatest in weaning and immediate post-weaning periods. Apart from the effect on growth mild and moderate protein deficiency in low income groups in the developing countries render young children susceptible to respiratory and gastrointestinal infections as well as to the retardation of intelligence. The incidence of such diseases causes mortality 20 to 50 times higher in 1 to 4 years age groups.

Pregnant and lactating mothers form another vulnerable group in whom the low protein intake may manifest in different ways. There is some evidence that a relationship exists betwen low birth weight and low protein intake of mothers. Pregnancy is followed by lactation which involves increased loss of protein from the body and consequently an additional protein requirement. Due to the established fact that protein and amino acid composition of milk from malnourished mothers are not significantly different from the milk from well nourished mothers, the protein need for milk secretion must be derived from mother's own tissues and consequently from her dietaries.

In contrast to young children and mothers the adults show a marginal deficiency which is sometimes seasonal and sometimes complicated by chronic diseases.

PROTEIN SUPPLIES AND DISTRIBUTION

Two sources of information are drawn upon to assess the current protein situation. The first one is the construction of Food Balance Sheets (FBS) by FAO[1] covering 90 per cent of World population and the second is the family or household food consumption surveys[2,3]. FBS shows average available

supplies of calories, protein and fat per caput per day in the country for the particular year derived from the production, import, export and disposal of available supplies. Whereas food consumption surveys shows actual intake of calories, protein and other nutrients through consumption of different food groups in different socio-economic groups living in different ecological regions of the country.

The available supplies of proteins (animal, vegetable and total) in grammes per caput per day in India and in different regions of the World are given in Table 1.

TABLE 1

Energy and Protein Supplies (1963-65) per caput per day.

Regions/Sub-Regions	Calories	Animal Protein	Vegetable Protein	Total Protein
Far East : South Asia	2050	8.6	46.2	54.8
East Asia	2350	20.5	64.6	75.1
India	1950	6.2	46.8	53.0
China (Mainland)	2010	8.2	50.5	58.7
Near and Middle East	2410	14.0	57.6	71.6
Africa	2170	10.9	47.6	58.5
East/South Africa	2270	15.0	49.8	64.8
Latin America	2590	24.1	43.5	67.6
Developing Regions	2140	10.7	46.9	57.6
Europe (incl : U.S.S.R.)	3050	42.8	44.8	87.6
North America	3140	65.3	27.8	93.1
Oceania	3230	63.9	31.5	95.4
World	2380	21.0	45.1	66.1

In Table 2 are given the percentage contributions of main food groups to total protein supplies.

TABLE 2

Percentage contributions of various commodities to protein supplies

Regions/Sub-Regions	Cereals	Pulses, Legumes	Vegetable source	Meat, Fish, Eggs	Milk	Animal source
Far East : South Asia	59.1	18.0	84.3	11.9	3.8	15.7
East Asia	48.2	14.0	72.7	24.6	2.7	27.3
India	59.0	27.0	88.0	2.0	10.0	12.0
China (mainland)	57.8	20.3	86.1	13.2	0.5	13.9
Near/Middle East	67.8	6.7	80.4	10.1	9.5	19.6
Africa	54.7	15.7	18.4	9.8	4.8	18.6
East/South Africa	55.1	15.6	76.9	16.5	6.6	23.1
Latin America	39.8	16.9	64.3	22.9	12.9	35.7
Developing Regions	57.2	16.8	81.4	12.9	5.4	18.6
Europe (incl : U.S.S.R.)	36.8	3.8	51.5	29.8	18.8	48.5
North America	17.6	4.6	30.1	45.0	24.9	69.9
Oceania	24.9	2.4	3.6	74.1	22.5	66.9
World	47.9	12.1	68.2	20.7	10.9	31.8

It is observed that in the developed countries the daily per caput protein intake comes to roughly 90 gms, in the developing countries the average is 57 gms (in the range 50-75 gms) and in some countries of South East Asia it is barely over 40 gms. The discrepancies are wider for protein from animal sources, 40-60 gms (av. 48 gms) in the developed countries, whereas in the developing regions 6-20 gms (av. 10.7 gms). In the latter cereals contribute 57%, 68% in the Near and Middle East, and pulses 16.8%. Owing to smaller amount of protein from animal products and diversity of supplies, the protein quality is unsatisfactory in the developing regions[4].

The problem is therefore unsatisfactory supplies both *quantitatively* and *qualitatively*. The quality depends on the two factors : *digestibility*, (DC) which measures the proportion of absorbed nitrogen of the dietary protein and the *Biological Value* (BV) which depends on the amino acid composition and measures the absorbed nitrogen that is retained in the body. The product of these two factors or the porportion of nitrogen of food consumed that is retained in the body is *net protein utilisation* (NPU) value of the dietary protein and is the most useful indicator of the nutritional value of dietary protein. The term NPU operative (NPUop) is mostly used which refers to the utilization of protein under those conditions in which it is actually eaten[5]. As a further step in the practical evaluation of diets, efficiency and concentration may be combined in a single index, which has been called the *the net dietary protein value* (*NDpV*) introduced by Platt and Miller[7]. This expression is obtained by multiplying protein concentration by NPU determined at the protein level.

Protein requirement

The protein requirements in a given country are calculated according to the recommendations of Expert Group of FAO and WHO on "Protein Requirements"[6] depending on body weight and population breakdown according to age and sex. These protein requirements are stated in terms of hypothetical ' Reference Protein ' (NPU 100) and then expressed in terms of ' *Local Protein* ' taking into account the operative Net Protein Utilization (NPUop) in the country in question. FAO has determined the NPU value of dietary protein in India which is 53%[4]. The protein requirements are shown in Table 3.

However, according to the field studies when the protein supplies of the population meet with the mean requirements or the supplies and the requirements are marginal, the requirement of about 40 per cent of the population of the country are not covered by their supplies. In order to cover the requirement of the great majority of the population (97.5%), it has been proposed by the Joint Expert Group[6] that the available supplies should be 120% of the

mean requirement. This is considered as '*Practical Allowance*' or the Recommended Allowance on the basis of which the production prospects and food policy should be based (Table 4).

Farthermore, to fulfil the quantitative aspect of protein requirement, the calories derived from dietary protein should be equal or above 10% of the available total supplies of protein (Table 4).

TABLE 3

Protein requirement

Gm per Kg. body weight per day

Age (months/years) Both sexes	Reference Protein (120% of Mean) Practical Allowance	Local Protein in India NPU 53%	Total Protein
Infants :			
3-6 mths ..	..	..	1.8
6-9 mths ..	1.5	1.5/0.53	2.8
9-12 mths ..	1.2	1.2/0.53	2.3
Children :			
1-3 years ..	1.06	1.06/0.53	2.00
4-6 ,, ..	0.97	0.97/0.53	1.83
7-9 ,, ..	0.92	0.92/0.53	1.73
10-12 ,, ..	0.86	0.86/0.53	1.62
Adolescents :			
13-15 ,, ..	0.84	0.84/0.53	1.58
16-19 ,, ..	0.77	0.77/0.53	1.45
Adults :			
All Ages ..	0.71	0.71/0.53	1.34

Example : Dietary protein requirement of Indian adult : 65 Kg man—87.1 gms
50 Kg woman—67.0 gms.

TABLE 4

Available local protein and protein requirement per caput per day

Regions/Sub-Regions	Supplies		Requirement			Protein Calories
	Calories	Total protein	Calories	Total protein		
				Mean	Pract.	
Far East : South Asia	2050	54.8	2250	49.0	59.0	10.7
East Asia	2350	75.1	2170	41.9	50.0	12.8
India	1950	53.0	2250	49.0	59.0	11.0
China (mainland)	2010	40.7	2370	52.5	75.0	8.0
Near/Middle East	2410	71.6	2410	56.0	72.0	11.8
Africa	2170	58.5	2250	52.0	62.0	10.8
Latin America	2590	67.8	2360	53.0	68.0	10.5
Developing Regions	2140	57.6	2270	50.0	61.0	10.8
Europe (incl : USSR)	3050	87.6	2690	50.0	88.0	11.4
North America	3140	93.1	2520	61.0	93.0	12.0
Oceania	3230	95.4	2700	56.0	95.0	11.8
World	2380	66.1	2370	53.0	66.0	11.1

Protein Gap

The following are some of the reasons for the marked difference in the supply and the requirement of protein :

(*i*) The marked difference in the per capita food production between the developed and developing countries is increasing because the death rate is declining, birth rate remaining high and the crop yield still low in the developing countries. In India, inspite of all the efforts on family planning 14 million people are increasing every year. The production of cereal crops increased to meet the needs but that of other essential food items showed no immediate prospect of meeting the needs.

(*ii*) While the *quantity* of food in terms of Caloriesis already causing concern in many areas through the developing world, the *quality* (notably protein) of the food consumption pattern is even more critical. The protein-calorie balance of the diet of over one-third of the population of the developing world is inadequate.

(*iii*) The gap between the nutritional requirements and the actual consumption of nutrients particularly of protein is widening rapidly with greater impact on young children—in many countries, one-third die before reaching school age.

(*iv*) Most developing countries are achieving significant increase in food production but the per capita food levels are declining as the population growth rates exceeds the rates of increased food availability.

Besides non-availability of adequate amounts of protein-rich food to meet the needs of the growing population there are several other reasons too :

(*i*) Inadequate knowledge of processing, handling and marketing operations, irregular deliveries and more wastages.

(*ii*) Even the available protein foods are not distributed evenly in different regions of the country and not even among the members of the same family.

(*iii*) Habits, prejudices and tabboos against certain foods.

(*iv*) Lack of knowledge of incorporating protein-rich foods in the daily meals of the family and lack of knowledge of nutritive value of locally available foods. Animal proteins are most expensive.

(*v*) Income and family sizes affect protein consumption.

World Food and Population Problem

Taking 1965 as base year, the food production between 1965 to 1985 should increase 2¼ times to keep pace with the population growth, 70% of which will account for the population growth and 30% for increased icome. Techni-

cally the policy is feasible but too difficult for meat, milk and such other essential food item. Furthermore, the production and commercial policies of rich countries is major handicap to the progress of poorer countries since they must have cash income and must export to earn foreign exchange.

The question therefore arises whereform this extra food will be availaale. Should these be shipped to the developing countries from the producing countries like North America, Western Europe, Australia and New Zealand. It is the official view of FAO that this extra food should be produced in the country since the problems will not be solved by supplying from the developed countries —need for rapid increase in food production incentive in the developing countries.

In 1962 food import costed the developing countries 3 billion dollars. If increased demand is to be met by import, it would cost 40 billion dollars in 1985. Secondly, it is hardly possible that the developed countries would be agreeable to give food aid on a permanent basis. Thirdly, the agricultural people must find employment, otherwise they will migrate to cities.

Projection into 1975 and 1985 for India

It is necessary to set close future dates when such requirements will be covered. The Indicative World Plan of FAO' set 1965 as the base year and adopted 1985 as the horizon year, with an intermediary stage in 1975. The next was to take into account individual requirements and population growth to determine the production goals for these two horizon dates. This was done country by country. The case of India is presented here.

Considering the population growth projected into 1975 and 1985 (using the medium variant of the United Nations assumption), it follows that 50 to 90 per cent higher protein supplies will be necessary by these dates. Obviously, that fraction of the population whose requirements are already covered will grow in number and its consumption will continue to increase or be of better quality. Assuming for this segment, a 2% rise in income per year and elasticity of protein demand of 0.3 (it is 0.55 for all of India), the total increase in protein supplies will have to be 60 per cent by 1975 and by 95 per cent in 1985.

Table 5 presents the indices of protein supplies (increased production of protein foods) required by 1975 and 1985 to cover Practical requirements (P) as defined as 120% of Mean requirements (M). This Table also gives indices of production of foods required to attain the objective (O) of caloric supplies

by the same dates, as compared to the supplie available in the base year of 1965.

TABLE 5

Increase of protein and energy supplies required by 1975 and 1985 to meet protein and energy targets

Regions/Sub-Regions	Calculated as 120% (P) of Mean protein requirement		Calculated as 10-12% (O) of Mean calorie requirement	
	1975	1985	1975	1985
Far East : South Asia ..	135	167	155	192
East Asia ..	115	133	115	133
India ..	153	193	163	207
China (mainland) ..	126	153	147	179
Near Middle East ..	131	172	131	172
Africa ..	135	176	146	190
North Africa ..	160	216	149	196
Latin America ..	134	177	140	185
Developing Regions ..	134	170	152	192
Europe (incl : USSR) ..	114	124	109	118
North America ..	115	135	115	135
Oceania ..	124	153	124	153
World ..	122	151	136	170

Although efforts may be made in various ways to meet and close the protein gap, the only subject to be discussed here is increasing of available supplies of protein foods—*proteins of vegetable origin.* Cereals constitute staple food of 95% population in the developing countries. Here are the percentage contributions of cereals to protein supplies in the principal developing countries of the World.

Brazil—38%	Africa—55%	North Africa—70%
Near East—68%	India, Pakistan, Ceylon—65%	Third world—58%
Eastern Europe—50%	Mexico, Centr. America—45%	Entire World—48%

Hence the principal method of increasing production of crude protein is to promote the production of cereals. Although the protein-calorie % of most of the cereals exceeds 10%, their NPU is only between 50 and 55%. It is therefore necessary to improve the quality of the diet by simultaneous production of certain quantity of protein foods other than cereals (pulses, animal products) for supplementation.

It is necessary to pay special attention to the protein quality of selected seeds. A variety of maize known as Opaque 2 (high lysin high tryptophane variety) has already been produced. There are limiting amino acids in these cereals too. Analogous results have been obtained both in Philippines and in Guyana by producing I.R.8 variety of rice. The protein content of the rice (I.R.8) produced by Dr. Pawar in Guyana was 11-12%, these were high-yielding short

variety. Mexico was pioneer in producing high yieiding wheat. The results of supplementation are shown in Table 6.

TABLE 6

Influence of protein value by supplementation with high yield/high protein varieties of cereals expressed as per caput per day

Country (Family consumption)	Total protein of diet g./day	Protein calories %	Limiting Amino acid	NPU %	NDp Cal %	Net protein g./day
IRAN :						
(1) Common wheat (Pr. 10.4%)	72.4	10.6	Lysine	55	5.8	39.8
(2) Wheat, improved (Pr. 12.4%)	83.5	12.3	,,	51	6.3	42.6
(3) Family diet (1)+10% of (2)	90.4	12.4	Threonine	51	6.3	46.1
PHILIPPINES :						
(1) Local rice (Pr. 7.3%)	42.3	11.1	sulphur	59	6.5	25.0
(2) HYV rice (pr. 10.7%)	53.1	14.0	A.A. ,,	54	7.6	28'7
(3) Family diet (1)+10% of (2)	56.5	13.8	,,	55	7.5	31.1
GUATEMALA :						
(1) Common maize (pr. 9.5%)	72.0	12.6	,,	49	6.1	35.3
(2) High Lysine Opaque 2	78.8	13.8	Threonine	59	8.1	46.5
(3) Family diet (1)+10% of (2)	83.7	13.8	,,	59	8.1	49.4

It is possible to deduce from the results shown in this Table 6 that :

1. Higher protein content does not necessarily mean higher NPU of the diet (*see* Iran). The improvement is negligible in Philippines and in no way proportional to higher protein intake because of the poorer quality of the protein in the cereals.

2. A major increase (from 30 to 35%) in net protein level is obtainable without necessarily any increase in yields, merely through the improvement of protein quality (Opaque 2 variety maize) in Guatemala.

FINAL COMMENT

It is well known that the targets and objectives are rarely attained. For Indian even with most optimistic prospect for the rise of individual income, protein consumption in 1975 will rise by one-third and in 1975 only by two-thirds of the increase envisaged in practical requirement (P). According to the same calculations, another 25 years of sustained economic development would be necessary to attain a production level such that a great majority of the people will have an adequate protein intake. The Indicative World Plan of Agricultural Production reaches a pessimistic conclusions for most part of the world.

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'NEUROCHEMISTRY OF MALNUTRITION*

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Perinatal malnutrition produces life-long mental blight

The infantile malnourishment is outstandingly serious because infancy is the period during which malnourishment can produce highly damaging effects that may persist and be pathological in later years. Before children are out of their mothers' arms, they are mostly victims of malnourishment in India, Pakistan, Bangladesh, Biafra, Chile, Mexico, Puerto Rico and elsewhere throughout the world. Those who manage to survive may be doomed to life-long mental blight. Numerous experimental and clinical findings have pointed sharply towards the conclusion that malnourishment imposed during a critical period of development produces permanent changes in brain structure and function. Neurological abnormalities, such as apathy and lethargy, are prominent symptoms of severe malnutrition in children. Both specific and nonspecific electroencephalographic changes may occur (reviewed by Winick and Coombs[1]. Some important neurochemical changes that occur in malnourished children are discussed in this review. Some of thes changes have already projected high risks of retardation of mental development as a result of perinatal malnourishment. These changes have enormous implications for about 400 million children living in food-deficient underdeveloped areas of the world and growing on protein-calorie diets. These are all the more disturbing as far as the future of malnourished children of India is concerned.

Perinatal malnutrition decreases brain's cellular contents

The number of brain cells becomes final at birth. It is not therefore unlikely that dietary restriction, particularly that of protein, during gestation of mother, may result in some permanent reduction in brain cells. Zamenhoff and associates[2] at Los Angeles' University of California School of Medicine checked this point by carrying out an animal experiment with female rats maintained on 8% or 27% protein diet by pair feeding schedule for one month prior to mating and through entire gestation period. The investigators observed that the brains of newborn rats from the females on the 8% protein diet contained significantly less DNA and protein compared to the progeny of the females on the 27% proiein diet. Further, there were fewer brain cells, and

the protein content per cell was lower in newborn rats of protein-deprived mothers. In a more recent experiment, Enwonwu and Glover[3] fed either 5% or 18% protein diet to timed pregnancy rats obtained at 14 days of pregnancy. The pups were sacrificed on day 2, 10, 15 and 21 postpartum. At all stages cerebral contents of RNA, DNA and protein were lower in the malnourished pups than pups suckled by adequately fed dams. In the former, RNA, DNA and total protein increased 3.6, 1.4 and 7.0 fold respectively between the days 2 and 21 compared with increases of 4.5, 2.0 and 8.0 fold for the equivalent fractions in adequately fed pups. Further, concentrations of tyrosine, aspartic acid, glutamic acid, alanine and leucine were lower in the brains of malnourished than in control pups. Furthermore, uptake of ^{14}C-leucine into cerebral polysomes after 1 hour of incubation was lower in malnourished pups than in well-fed pups. Nutritional restriction during the period of rapid postnatal brain growth also decreases brains cellular contents. Howard and Granoff[4] subjected mice to nutritional restriction by intermittent removal from their mothers during the period of rapid postnatal brain growth at 2 and 16 days of age, using split litters. A 57% reduction in body weight was produced in 16 days. Thereafter, they were fed *ad libitum*. At nine months, body, cerebral and cerebellar weights were reduced by 17, 7 and 14% respectively, compared to litter mate controls. Total DNA was reduced 8% in the cerebrum and 22% in the cerebellum. Thus, quite mild malnutrition at the time of fastest brain growth brings about a lasting deficit in brain size, in the number of brain cells and their cellular contents. Nutritional improvement afterwards does little or nothing to correct the damage that has already occurred as a result of malnutrition at a critical period of development. In contrast, severe undernutrition applied after the critical period has less effects that are more rapidly reversible. There are aspects of brain growth which must occur at the correct chronological time and if this opportunity is missed due to nutritional or other restrictions, complete rehabilitation may not be possible. In rats and mice, the period of fastest brain growth is believed to be the first few weeks after birth. In man, the corresponding period of rapid brain growth is believed to be the last few weeks before birth. Clinical data point out that undernutrition in early human life results in the reduced brain size and weight[5].

Malnutrition lowers brain DNA

Because DNA content of cells is constant for each species, it is possible to determine the number of cells in a tissue sample by quantitating the DNA content. By plotting normal DNA values Dr. M. Winick of New York's Cornell University Medical Center demonstrated that cell division in human brain slowed at birth, but continued until about 6 months. Thus, good

early nutrition appears to be a key to normal division of human brain cells. Animal studies have established that lack of sufficient protein intake interferes with DNA synthesis and consequently with brain cell division [4, 5, 7]. Dr. Winick at the University of Chile at Santiago studied brains of infants less than a year old who had died of wanting disease (marasmus) and compared them with brains of normally nourished U.S. and Chilean infants who died of accidental causes between 13 weeks of gestation and one year of age. Babies who died of marasmus had 25% fewer cells in their brains than the well-nourished infants, and few had up to 60% fewer cells. In other words, the increases in DNA with growth was not found in brains of Chilean infants who died from marasmus—a disease of protein-calorie malnutrition. He also showed that damage to brain of a human infant is irreversible if malnutrition occurs before six months of age[8].

Malnutrition impairs learning behaviours and mental capacity

In a study at Johns Hopkins University at Baltimore difference in activity and emotional behaviour as well as learning behaviour were shown in progeny of mother rats restricted to 50% overall *ad libitum* dietary intake during gestation and lactation[9]. In an extended study, it was further demonstrated that, in contrast to the normal group, animals from mothers underfed during gestation were timid, and their normal social interaction was much inhibited[9]. In another animal study at Cornell University at Ithaca, it was demonstrated that early protein-calorie malnutrition resulted in long lasting changes in both food-related and non-food-related behaviour[10]. Winick showed in experimental animals that early malnutrition resulted in impaired learning ability[11]. Several retrospective studies in developing countries throughout the world in which careful control groups were chosen have suggested again and again that early malnutrition interfered with children's learning ability (reviewed Winick and Coombs[1]). Even infants in the U.S.A. who were severely malnuurished in life performed consistently poorly when subsequently tested at a later age[12]. In September, 1968, Dr. P. Monckbery of the Department of Pediatrics and Nutrition of the University of Santiago, Chile presented evidence at the Second Western Hemisphere Nutrition Congress that brain growth and mental capacity of infants subject to severe protein malnutrition from the first month of life were markedly impaired and an intermediate state of malnutrition produced damage of an intermediate nature[13]. According to the evidence presented by Dr. F. Beas of the University of Chile at the 5th Annual Uniform Services Pediatric Seminar at West Point, New York, the protein-calorie restriction produced effects in mental and motor development that are more severe the earlier in life the deficiency started[14].

Malnourished children have poor I.Q.

A group of 14 infants admitted to metabolic wards of Dr. Beas' Santiago hospital with severe marasmus were found to have height and cranial circumference measurements under the third percentile after some years of follow-up. Binet testing showed the present intelligence quotients (I.Q.) of these children averaged only 62. In a retrospective analysis, Cabak and Najdanvic[13] demonstrated that Serbian children with a history of marasmus had significantly lower I.Q. than Serbian children in general. Stoch and Smythe[16], studying South African children, showed previously that those malnourished early in life were smaller than a control population and had reduced head circumference and I.Q. even after long-term follow-up. Cravioto and other [17-19] studied populations of uniform socio-economic backgrounds in Mexico and Guatemala and found that performance on psychological tests was related to dietary practices and not to differences in personal hygiene, housing, cash income, crop income, proportion of income spent on food, parental education and other social or economic indicators. Further they found that earlier the malnutrition, the more profound is the psychological retardation, and that the most severe retardation occurred in children admitted to hospitals under 6 months of age. In Cravioto's studies in Mexico, infants, victims of malnutrition in their first six months, faced the possibility of never catching up despite later adequate feeding, and there was a tendency for malnourished infants to remain intellectually deficient in comparison with their siblings[14]. In terms of audio-visual performance and height, they never quite advanced to the point where they should be relative to their chronological age. According to Cravioto[14], height —a biological measurement of difference in nourishment—appears to correlate with the power of reasoning and intellectual achievement. Few years back, Dr. Rene Dubos of Rockefeller University studied the effects of early dietary deprivation on children in Guatemala. Indian infants in poor districts in Guatemala are raised on a diet rich in corn and poor in animal protein such as that in milk. When these children were taken at five and placed in special school with a well balanced diet, they remained backward. Seemingly, irreparable damage has been done to them in their early years. However, in Cravioto's studies [17-19] children admitted later with the same socio-economic backgrounds and the same severe malnutrition but at a later time of onset didperform better in psychological tests with prolonged rehabilitation. Much earlier in 1954, Kugelmass and associates [20] demonstrated that rehabilitation in a group of New York children malnourished later in childhood significantly increased their I.Q. Thus, the time of onset of malnutrition rather than malnutritional state may be of great importance in determining whether or not the intellectual retardation will be permanent.

Does malnutrition produce chromosomal damage ?

Incidently, the above question was raised in a study in Mexico by Armendares and associates [21], who studied 5 children with kwashirkor and 5 with marasmus. Aged one month to 5 years, these children were all severely malnourished with body weights 37-54% less than the ideal weights. The investigators carried out cytogenetic studies on the day of admission into hospitals and serially thereafter, and examined their bone morrow. According to their reports, (*i*) chromosomal abnormalities were approximately six times more frequent in cells from the malnourished children, (*ii*) there was no consistent decrease in percentage of chromosomal abnormalities in malnourished children up to 60 days after initiation of rehabilitation therapy, (*iii*) chromatids had 178 abnormalities such as gaps, isogaps and breaks in cells from malnourished children and only six in those from controls, (*iv*) malnourished cases showed serious abnormalities such as decentrics and acentrics, and (*v*) chromosome analysis of cells of the bone marrow showed 33% abnormalities. Further, seven children were reexamined after 1 year when they achieved normal weights for height. At this time, chromosomal abnormalities were still four times more common in their cells than in those of controls. These observations of malnutrition-induced chromosomal abnormalities which appear to persist have raised questions regarding the consequences of these abnormalities on physical and mental health of the victim.

Underfed children retard socio-economic developments

The findings cited in this review tend to project high risks of retardation of physical and mental development in perinatal malnutrition. In parts of the world where the majority of infants are malnourished because of socio-economic reasons, the limitation of physical health and mental capacity means, in the end, an impossibility of improving socio-economic developments. A backward country where infants are underfed or malnourished is almost doomed to remain a backward country. " Not only are they (malnourished children) to be handicapped, but the resulting handicap may well prevent them from extricating themselves from the conditions which breed malnutrition, and therefore their children are at risk of suffering the same handicap. Thus, poverty results in undernutrition which causes retarded brain growth and function which limits earning capabilities which lead again to undernutrition. This is a vicious cycle first pointed out by Cravioto [17] " (quoted from Winick and Coombs [1]). An over-populated nation such as India is likely to face a terrible situation in the next decade when a substantial portion of her growing children will show signs of retarted physical and mental development as a consequence of malnourishment in their infancy today. This will be an additional

burden on the developing Indian nation if proper remedy is not sought today. While India is making some efforts in restricting her population growth, which should be more drastically checked, it should be, at the same time, the nation's foremost responsibility to ensure proper and adequate nourishment to today's infants. There is no blanket solution for the problem of malnutrition except assigning priority on infant's food and nutrition. While millions of Indian children are victims of malnourishment day in and day out, India is destined to crop physically and mentally deficient citizens in near future unless she first feeds her hungry and malnourished children.

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RAUWOLFIAS : BIRTH OF A NEW DRUG FROM AN OLD INDIAN MEDICINAL

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The root of *Rauwolfia serpentina* Benth. (Sanscrit : 'Ahimardini' ; Hindi : 'Sarpagandha,' 'Ardhachandrika' or 'Chotachand' belonging to the Natural Order, Apocynaceae), a wild-growing plant in the tropics, has had a long and colourful record in Indian medical lore and local physicians employed it for centuries for the relief of a great variety of ills. This medicinal herb has been mentioned in several of the renowned treatises on Hindu Medicine, as a remedy for snake bites and insect stings. It is also mentioned in ancient folk literature as a febrifuge and a cure for dysentery. Its reputation extended to Europe by 1563 (Gracia ab Horto) and there is authentic record of its use in 1690 (Rumphius). In the 'Herbarium Amboinese (1741), it was stated that Serpentina roots were "valet contra anxietatem," meaning a drug which can remove all 'anxiety states.' In comparatively modern literature, the earliest reference to the medicinal uses of Rauwolfia serpentina dates as far back as 1891 (Dymock, W.). It was considered as "a foremost and most praiseworthy of Indian medicine," and is said to cause uterine contractions and has been recommended as an ecbolic. The roots of the plant gained a reputation of being a general sedative and a cure for certain types of insanity, hysteria and epilepsy. In parts of Bihar, the root powder is sold in the market as 'Pagal-ka-Dawa' (insanity cure drug).

The drug owes its name to the German physician and botanist, Leonhard Rauwolf, who towards the end of the sixteenth century made an extensive tour in Asia and Africa in quest of medicinal plants mentioned by early Greek and Arab physicians with a view to investigate them and published an account of

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†Dr. B. Mukerji had been an intimate scientific colleague of late Prof. B. C. Guha and had been engaged in pharmacological and biochemical researches on Indian indigenous drugs at Calcutta more or less during the period of Prof. Guha's active association with protein and vitamin researches in the Calcutta University. This article is a review of Dr. B. Mukherjee's contributions (in collaboration with Chopra School of drug research workers) to the Rauwolfia problem, which had played a significant role later in the introduction of a modern drug from ancient Indian materia medica.

his travels in 1583. A French Botanist, Plumier, 1903, named the plant *Rauwolfia serpentina* in honour of Dr. L. Rauwolf.

The genus of Rauwolfia is widespread in the tropics in India, Burma, Ceylon, Java, South Africa, Brazil and other places and nearly 131 species are known to exist, of which 8 are reported to occur in India. Some of the species already examined have been found to contain alkaloids and many are said to possess valuable medicinal properties. Only about a dozen or so of these, *viz. Rauwolfia serpentina* Benth, *Rauwolfia canescens* Linn., etc. are commonly available and the information regarding the pharmacognosy, chemistry, pharmacology, etc., of the rest are extremely meagre. Amongst these, *R. serpentina* Benth, is the most important and grows fairly widely in India and Pakistan, the Andamans,, Ceylon, Burma, Cochin, China, Malaya, Java and the Phillipines. In India it occurs in the moist and hot parts of the sub-Himalayan regions, Rohilkhand, Oudh and Gorakhpur, also in Konkan, N. Kanara, S. Mahratta, the eastern and western Ghats of Madras State upto 3000 ft., in many districts of Bihar and in North and Central Bengal. It is a small erect glabrous shrub with flowers, white to pale rose in colour and with fruits which are purplish black at maturity. Several sub-varieties of Rauwolfia and *R. canescens* grow in the same areas as *R. micrantha*, another species grows in Malabar coastal plain and *R. densiflora* in the Khasia mountains, Assam. So far these drugs have been collected from wild sources and from isolated areas in the Himalayan foot-hills.

Experimental cultivation of *R. serpentina* has been started recently in several suitable areas. Reports indicate that it can be cultivated from seeds as well as from stem and root cuttings in the plains, in evergreen forests and the sub-Himalayan tracts. In pot plant experiments in the Central Drug Research Institute, Lucknow, about 30% germination from fresh seeds was recorded, which was extremely poor. The alkaloid content of the roots of the plant grown at higher altitude has been found to be greater than those grown at sea level. The Forest Research Institute, Dehra Dun, has shown that it is possible to obtain a yield of 2,000 lb. of high quality roots per acre in two to three years.

Early Therapeutic History :

Until about 1929-30, its action on blood pressure was generally unknown and modern doctors were ignorant of its value as a sedative, although many Indian mothers used the powdered root in infants for its calmative effects. During this period, a Patna physician (P. C. Ray, 1931) and two Calcutta Physicians, Kaviraj Gananath Sen and Dr. Kartikchandra Bose (1931), after prolonged clinical trials on the drug for several years, announced that the root-powder of Rauwolfia not only produced sedation and hypnosis but also caused significant lowering of high blood pressure. At about the same time, Hakim Ajmal

Khan of Delhi, another modern doctor interested in the study of ancient Arabic and Unani medicine, also observed independently the good sedative and blood pressure-lowering effect of the drug. He was so impressed with the virtues of this remedy that he induced two modern organic chemists, Siddiqui and Siddiqui (1930-30) to undertake a systematic investigation of the chemical composition of the drug. Almost simultaneously, at the request of Sen and Bose, R. N. Chopra at the Calcutta School of Tropical Mecicine, started a series of chemical and pharmacological investigations on Rauwolfia, in collaboration with S. Ghosh, the noted plant chemist. The Delhi chemists isolated 5 crystalline alkaloids, and named them as (1) Ajmaline Series : Ajmalinine, Ajmalicin, and (2) Serpentine Series : Serpentine, Serpentinine. The Calcutta group produced a total alcoholic nitrogenous extractive and also an alkaloid or a mixture of alkaloids with a m.p. of 135°. These active principles supplied the pharmacologists with the material needed for their modern pharmacological investigation.

Early Pharmacological Investigations :

After more than one year's laboratory work, the first paper was published by Chopra, Gupta and Mukerji in 1933 in the Indian Journal of Medical Research. The conclusions arrived at by the workers on the basis of the pharmacological evaluation methods then available are recorded below :—

" The alkaloid (from *R. Serpentina*) has a stimulant effect on ihe plain muscle of the intestine and the uterus."

" The systemic blood-pressure falls due to dilatation of the blood vessels of the splanchic area. The respiration is depressed death occurring from failure of respiration due to the paralysing effect of the alkaloid on the respiratory centre."

" The alkaloid has a pronounced effect on the central nervous system. In sub-lethal doses injected into the lymph sac of frogs narcosis quickly ensues. In mamamls the alkaloid produces symptoms which are attributable to its depressing effect on various cerebral centres in the reverse order of their development. The short period of exictement seen in guinea-pigs and cats is probably due to the dissolution of the higher centres as is so often seen with morphine, chloroform and alcohol. There is also evidence to show that there is some depression of all nerve-cells in the body."

" The alkaloid on account of its cerebral depressant properties should prove to be a valuable sedative drug. Its depressant effect on the respiratory centre should, however, be borne in mind. It lowers the blood-pressure and if admlnistered in proper dosage should be of value

as a remedy against hyperpiesis. Purgation is usually produced by the drug on account of its stimulant properties on the plain muscles of the gastro-intestinal tract. Its stimulant effect on the uterine movements, both virgin and pregnant, coupled with its pain-relieving properties should be useful during parturition. The drug is likely to be a valuable addition to the armamentarium of physicians and further work to place it on a definite therapeutic basis is in progress."

In 1941, Chopra and Chakravarti made a comparative study of the three isolated alkaloids of the plant, namely, ajmaline, serpentine and serpentinine, obtained from Dr. S. Siddiqui of Delhi. In decerebrate animals ajmaline raised the blood pressure while in spinal animals there was fall, which was more marked if the blood pressure was previously raised through adrenaline infusion. They suggested that ajmaline was a central vasomotor stimulant but had depressant effect on the heart and peripheral vessels. The sum total of the effect was a rise of blood pressure in decerebrate animals because the vasomotor stimulation masked the peripheral depressant effect. When, however, the vasomotor centre was excluded, as in spinal preparation, the peripheral effect alone prevailed. The effect of this product on intact animals was not reported.

In 1942, Chopra, Bose, Gupta and Chopra obtained the three alkaloids from Siddiqui and investigated the comparative action of these alkaloids. They confined themselves to a study of the effect on blood pressure. They concluded that the chief preasure-reducing factor in the plant was serpentine. Although all the three alkaloids produced a fall in blood pressure after experimental hypertension, ajmaline and serpentine raised the blood pressure in intact normal animals. The crude extract also produced a fall in blood pressure. The hydrochloride of the total alkaloids from which ajmaline, serpentine and serpentinine had been removed had no effect on blood pressure. The alkaloids had both direct effect on the plain muscle of blood vessels and through the vasomotor control of the blood vessels. These results were more or less a confirmation of their earlier observations in 1941. At this stage it appeared that ajamaline and serpentinine belonged to one group in respect of their effect on blood pressure and intestine muscle while serpentine to the other. On the other hand, in respect of effect on the C.N.S., ajmaline and serpentine were stimulants and serpentinine was the sedative alkaloid.

In 1943, Chopra, Gupta, Bose and Chopra focused their attention on the hypnotic principles of the plant. As a result of this study they revised the earlier conclusion arrived at by Chopra and Chakrabarti regarding the sedative properties of serpentinine. This alkaloid was found to be a C.N.S. stimulant similar to ajmaline and serpentine. Regarding the nature of the hypnotic and

sedative principle of the drug, it was suggested that the alcohol-soluble fraction on fractions other than the three isolated alkaloids was responsible for this property of the plant.

In 1944, Gupta, Kahali and Datta reviewed the whole position relating to the hypnotic principle of *Rauwolfia serpentina*. These workers recorded that the oleoresin fraction, free from practically all traces of alkaloids known at that time, produced a sedative and hypnotic activity in cats, rabbits, frogs and guinea-pigs. The effect of this fraction was similar to that produced by the standard extract, commencing 3-4 hr. after administration and lasting for more than 24 hrs. These workers, therefore, just missed the chance of discovering 'reserpine' from the oleoresinous fraction, which was later worked upon by the Swiss workers, leading to the discovery of 'reserpine,' the most important alkaloid in Rauwolfia.

Early Clinical Observations :

Professor Chopra was a pharmacologist with a strong clinical bent of mind and as soon as the acute toxicity studies with the Rauwolfia alkaloid were completed in the laboratory and a dosage level safe enough for human administration could be worked out, he started out with human trials with both the standardized crude extract and the crude powdered materials from rauwolfia. On the patients under his care at the Carmichael Hospital for Tropical Diseases, Chopra and Gupta obtained satisfactory correlation of the data obtained in the Pharmacology Laboratory and he was so impressed with particularly the prolonged sedation effect of the drug, that he and Mukerji undertook, in collaboration with the Director of the Ranchi Mental Hospital in Bihar, a controlled clinical trial of the drug in several types of mental disorders showing violent maniacal symptoms. Results obtained in some cases of schizophrenia were encouraging but uniform recordable data which could stand the test of critical analysis were not available. In one set of trials, actually a recrudescence of violent symptoms with increased agitation and excitement was recorded. In view of the inconclusive nature of the data, Chopra discouraged the publication of the data obtained from these human trials at that time. Meanwhile, clinical trials with the Rauwolfia drugs were confirmed outside the Hospital and through his influence Chopra was able to get reports of these cases treated by private physicians outside the regular hospital clinics. An overall analysis of the data obtained from these sources left little room for doubt that Rauwolfia drug was a definite contribution to therapy, particularly in cases with high blood pressure and mental agitation symptoms.

Since the reported efficacies of the whole powdered drug in hypertension and insanity by Sen and Bose, many workers started investigation along the same

lines and used either whole powdered roots, or extract or individual active principles either alone or in combination with other drugs and have unanimously found the drug to be effective in hypertension as well as in deranged mental conditions. Dr. Bhatia of Lucknow and Dr. Vakil of Bombay have also corroborated these results.

In the treatment of high blood pressure, the drug is well tolerated and according to Dr. Wilkins the effect of Rauwolfia is so gradual and easy that it brings about a slow but steady reduction without the body apparently being aware of it. The use of crude preparation in American patients were associated with undesirable side effects such as drowsiness, lassitude, and diarrhoea although it has been considered as effective in types of hypertension and even superior to the hypotensive agents in several clinics in Germany. Unlike most blood pressure reducing agents, Rauwolfia preparations do not lead to habit formation. A systolic pressure drop of over 30 mm. and a disstolic of over 15 mm. has been observed and the pressure effect was gradual and attained its maximum after 2-3 weeks. With crude total alkaloids, the hypotensive response is less marked and takes longer time. Isolated individual alkaloids like R.S.51, 'hypotensin,' 'reserpine' have also been tried and found effective. Recently combined effect of *R. serpentina* with other potent hypotensive agents like hexamethonium have been proved to give even better results.

Early Chemical Investigations :

The world-wide interest in this Indian drug has led to a detailed chemical examination of its constituents in India, Europe and America. Already about 27 alkaloids have been isolated from *R. serpentina*, 2 from *R. canescens*, 3 from each of *R. caffra* and *R. vomitoria*, 2 from *R. obscura*, 1 from *R. semperflorens*, 4 from *R. heterophylia* and several other unidentified bases from *R. natalensis*. Some of these alkaloids are common in nature and some have already been proved to be of considerable therapeutic importance. The roots of *R. serpentina* contain the maximum amount of the alkaloids and the best sample of the roots has been found to contain a total alkaloids of 2.7% with a reserpine content of 0.09%. In the case of *R. canescens*, another important Indian species containing hypertensive principles, the alkaloid is mostly present in the leaves and its content varies with the season, being amximum in October-January when it reaches 1.2%.

(1) *Discovery of Reserpine :*

A quotation from the paper of Muller, Schittler and Bein will be appropriate in this connection :

" ....Since we had been concerned with Rauwolfia alkaloids for some time we made it our task to isolate the active sedative component of

crude Rauwolfia extracts. An attempt of this kind had been made previously by Indian workers, but they did not manage to improve on the crude "oleoresin fractions." Starting with these fractions we isolated the sedative principle in a pure, crystalline form. The substance obtained by us in a relatively week basic alkaloid with a melting point of 262-263° and a rotation (α) D of—117 to—118° (in absolute chloroform). We named the new alkaloid *reserpine*."

"The predominent pharmacological action of reserpine is a pronounced long lasting sedation of central origin. Even low doses (0.1 mg/kg, given i.v. to rabbits, and 1 mg/kg, given orally to dogs) produce peaceful sleep lasting several hours in animals. A noticeable feature of the action of reserpine is that the animals can be roused even after high doses have been given."

(2) *Failure of the Calcutta workers to isolate Reserpine :*

It is now possible to look back and speculate on the failure of the Calcutta group of workers to isolate reserpine, the principal active alkaloid of Rauwolfia, inspite of the fact that they were earliest to recognise the merits of this wonder drug and spent nearly 15 years of investigative work trying to put it in the armamentarium of the modern doctors. Rauwolfia, like most plant products, presented a complex mixture of strong and weak base alkaloids and separation of active constituent from such a mixture was not an easy task.

The alkaloids are usually isolated by taking advantage of their basic character. The crude total extract, after evaporation of the solvent (alcohol) is extracted with very dilute acid which dissolves the alkaloids. From the clear acid extract the alkaloids are obtained after basification. In the case of roots of *Rauwolfia serpentina* a similar procedure was adopted. After extraction with very dilute acid, substantial amounts of a deep brown resinous mass was obtained which was insoluble in dilute acids and hence in those days regarded as 'oleoresins' free from alkaloid and were discarded.

This 'oleoresin' fraction was found to have much stronger scative action than the acid soluble crystalline alkaloids. In fact, the sedative action characteristic of *Rauwolfia serpentina* appeared to be concentrated in the so long discarded 'oleoresin' (J. C. Gupta, S. Ghosh, A. T. Datta and B. S. Kahali, 1947 ; A. T. Datta, J. C. Gupta, S. Ghosh and B. S. Kahali, 1947).

These observations of the workers at the Calcutta school were duly utilised by J. M. Muller, B. Schlittler and H. J. Bein (Experientia, 1952) in working out a special method for isolation of the weakly basic Rauwolfia alkaloid, reserpine. It was shown that the typical pharmacological properties of Rauwolfia, as

observed in the 'oleoresin' by workers at the Calcutta School of Tropical Medicine, was due to reserpine. This weakly basic crucial alkaloid eluded the workers at CSTM in view of its insolubility in dilute acids, which was mostly used in those days for extraction of alkaloids from neutral products in the total plant extract.

Concluding Remarks :

Both the therapeutic virtues of R. serpentina—its sedative action on the central nervous system and its blood pressure lowering property—were recognised and regarded by early Indian physicians and it is interesting to note that these findings are being supported with modern work both with crude drug as well as its chief active principle, reserpine. In mental aberrations and constant nervous tension states, it is becoming clearly established that in this Indian drug, psychiatrists have got a remedy which, though slow acting, offers a sedative, in many ways superior to the frequently used barbiturates. The great wave of enthusiasm for the use of the Rauwolfia alkaloids in psychiatry came during the mid-1950s. Their sedative action was characterized as 'tranquilization,' a label that has since been applied to a variety of other compounds with similar sedative effects.

The drug is not a new discovery of the western countries but is a gift to them from India where it was known from centuries ago. Dr. Wilkins at Boston has rightly remarked "we owe a great debt to our colleagues in India. In the *Rauwolfia* drugs, they have given us a new approach in treating some old diseases which are very common and very distressing." Several other reports are unanimous in the belief that in this rediscovery of an Indian drug a long sought-for remedy has been found to treat conditions for which no other safe and effective treatment could be found in European medicine, till the Synthetic drug, chlorpromazine came into the picture in late 1950s. Painstaking research into ancient materia medica of India, China, etc. with the tools and techniques of modern medicine may result in the rediscovery of other worthwhile drugs as Ephedra and Rauwolfia etc.

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STUDIES ON VITAMINS, ENZYMES AND THE LIVING SYSTEM

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Highlights of some of the results on Vitamin C metabolism in animals and Vitamin B_1 metabolism in microorganisms and some related observations carried out in this laboratory have been presented. Some of the projects were undertaken by the author with initial inspiration from Late Professor B. C. Guha.

VITAMIN C METABOLISM IN ANIMALS

After the elucidation of the pathway involved in the biogenesis of Vitamin C in the animal system mostly through the efforts by Guha and his associates (1-8), Lehninger and his associates (9, 10), King and his associates (11-13), Mapson and his group (14-16) and Shimazono and his associates (17, 18) extensive investigations have been carried out in this laboratory to study the interrelationship between protein nutrition (19-23), mineral nutrition (24-28) and Vitamin C metabolism in animals and also on the hormonal regulation of some of the enzymes involved in the metabolism of Vitamin C (29-32). The contributions arising out of such studies are described below in brief outline :

(*i*) *Effect of protein nutrition on Vitamin C metabolism*

Studies have been carried out in detail on the interrelationship of dietary protein of varying quality such as casein, groundnut protein and soybean protein and Vitamin C metabolism to understand the metabolism of this important vitamin under protein sufficiency and deficiency conditions (19-22). The importance of protein nutrition and the effects of starvation and subsequent repletion with protein in the biological control of the synthesis of enzymes involved in the Vitamin C metabolism in rats have also been stressed in our studies (23). The content of protein in the diet was varied from 2-60% with rats as experimental animals. In the case of groundnut protein and casein the level of 10-20% was found to be optimum for maintenance of tissue level of this vitamin. The activities of *D*-glucuronolactone reductase and *L*-gulonolactone oxidase increased with increasing amounts of dietary protein till

9% casein level, and the activities remained stationary thereafter upto 60% casein level. But dehydroascorbatase activity was found to be increased markedly at 2% casein level and a further increase in dietary casein did not increase the activity. Uronolactonase activity increased with increase in dietary protein content upto 18%, and xylulose biosynthesis was found to be not at all affected by dietary protein content (19).

Studies carried out using guineapigs, the species which can not synthesize Vitamin C in their system, showed some interesting variations in the levels of the enzymes involved in the metabolism of Vitamin C, as compared to those of the rats, when they were maintained on high or low protein diet in normal and in scorbutic condition. The activity of liver dehydroascorbatase decreased slightly in guineapigs fed higher amounts of casein. This was more marked in the case of scorbutic animals. This can be explained by assuming that the animals fed higher amounts of protein try to conserve more vitamin in their tissues. Xylulose biosynthesis increased significantly in the case of normal and scorbutic guineapigs fed higher amounts of protein (21).

(*ii*) *Effect of metal ions on Vitamin C metabolism*

The role of metal ions on Vitamin C metabolism in animals has also been studied extensively (24-28, 33). Administration of molybdenum, manganese and cobalt decreased the concentration of Vitamin C in liver and kidney of rats (24, 25). Cr^{+6} or W^{+6} at a dose level of 5ppm resulted in an increase in the tissue concentration of this vitamin (26). Feeding of minerals like Mo^{+6}, Co^{+2}, Zn^{+2}, Cu^{+2}, Cr^{+3} or W^{+6} reduced the urinary excretion of this vitamin (24-26, 22). The synthesis of Vitamin C was found to be inhibited by Mo^{+6}, Zn^{+2} and Cu^{+2} and increased by Co^{+2}, Cr^{+3} and W^{+6} and unaffected by Mn^{+2} (22, 24-26). Dehydroascorbatase activity was found to be inhibited by Mo^{+6} and Cu^{+2} and increased by Mn^{+2}, Co^{+2}, Cr^{+3} and W^{+6} while Zn^{+2} was not found to affect the enzyme activity (22, 24-26). Except for W^{+6}, uronolactotase activity was found to be decreased by the administration of all the metal ions studied (24-26). Xylulose biosynthesis was found to be increased only with administration of W^{+6} (26). In scorbutic guineapigs the increased activity of the enzyme dehydroascorbatase, could be brought down to the control level, by the administration of Co^{+2}, Mo^{+6} and Mn^{+2} (28). While studying the effect of Cd^{+2} on the tissue reserve and metabolism of this vitamin it was found that there was marked decrease in the concentration of Vitamin C in the liver of rats under cadmium toxicity (27). The rate of Vitamin C synthesis was greatly reduced with an increased catabolic break down of this vitamin under cadmium toxicity. The enzyme of *L*-xylulose biosynthesis was markedly affected by cadmium toxicity.

(*iii*) *Interrelationship of Vitamin C and other vitamins*

It has been observed in this laboratory (34), that activities of the enzymes responsible for the biosynthesis of Vitamin C is decreased markedly in rats maintained on Vitamin E deficient diet. When necrogenic diet fed group of animals were supplemented with sulphur containing amino acids like *L*-cystine or *DL*-methionine, the inhibition of the above enzyme levels was largely overcome. Dehydroascorbatase and uronolactonase activity were found to be stimulated in group of rats maintained on necrogenic diet. Since Vitamin C metabolism is affected under Vitamin E deficiency conditions and also that Vitamin C is involved in the metabolism of cholesterol, studies on cholesterol metabolism were carried out under Vitamin E deficiency condition. The results indicate increased synthesis and simultaneous decrease in the catabolism of cholesterol under the experimental conditions (35). The hypo- and hypervitaminosis of A and D (36) and hypo-vitaminosis of several B vitamin (37, 38) on the status of Vitamin C metabolism in animals have been studied.

(*iv*) *Hormonal regulation of Vitamin C metabolism*

The enzyme levels in animal tissues could be altered by a wide variety of physiological, nutritional and hormonal manipulations. Several models have also been proposed on the mchanism of regulation of enzyme synthesis in animal tissues in respect of certain enzymes such as tryptophan pyrrolase, tyrosine transaminase, glucuronyl transferase etc. (39, 40). The effect of several drugs and also certain metabolic regulators on the enzymes involved in the metabolism of Vitamin C and of uronic acid pathway have also been widely studied during the recent years (41-43). The activities of some of the enzymes in this pathway have recently been found to be altered under various physiological conditions including that of adrenalectomy, alloxan diabetes, partial hepatectomy and administration of certain pharmacological substances (29, 30, 43-45). It is also known that Vitamin C plays an important role in the processes of corticosteroids metabolism in adrenals (39). It has been noted (30) that adrenalectomy brings about a drastic decrease in the level of liver gulonooxidase, with a simultaneous increase in the activity of dehydroascorbatase. Administration of hydrocortisone to the adrenalectomized rats can stimulate the activity of gulonooxidase appreciably and this stimulation is sensitive to Actinomycin D. This holds good also when untreated animals were given hydrocortisone injection. Recently a post transcriptional regulation of gulonooxidase by insulin has been suggested by Chatterjee *et al.* (29). The role of glucocorticoids as an inducer and of insulin as a repressor of key gluconeogenic enzyme is well established (46). It is also known that removal of adrenals in diabetic rats is effective in reversing the highly increased acti-

vities of the enzymes involved in the process of gluconeogenesis to a normal range in a few days and after adrenalectomy blood sugar level is also found to be decreased towards the normal level. Studies have been carried out on the effect of hydrocortisone on the enzymes involved in the metabolism of Vitamin C in alloxan diabetic and alloxan diabetic and subsequently adrenalectomised rats (43). At present it is not clear how the level of this important enzyme gulonooxidase is altered under conditions of various physiological and nutritional manipulation, but it could be clearly understood that insulin and hydrocortisone stimulate gulonooxidase and hence regulate the pathway of Vitamin C metabolism in rats in a different manner in respect of their site of action.

The synthesis and catabolism of Vitamin C and also the concentrations of several lysosomal enzymes are found to be altered by partial hepatectomy. Effects of various metabolic regulators like ACTH, glucagon, Cyclic AMP etc. under this condition have also been studied (47). Investigations on the effects of castration and subsequent administration of either testosterone or human chorionic gonadotrophin in rats provide some interesting information in relation to the regulation of gulonooxidase (31).

Biochemical studies on antimetabolite resistance in microorganisms

Extensive studies have been carried out on the metabolic alteration of *Staphylococus aureus* as a result of its adaptation to a potent antimetabolite 'pyrithiamine.' *S. aureus* needs thiamine for its growth and pyrithiamine inhibits it. When *S. aureus* is made resistant to pyrithiamine the organism becomes dependent on this antimetabolite. Several metabolic alterations develop in regard to the utilization of carbohydrates through glycolytic pathway, pentose cycle pathway and tricarboxylic acid cycle pathway (48, 49). Also there is alteration in the carotenogenosis (50) and amino acid utilization (51) of this strain. The strain is found to be lacking in respect of the enzyme β-galactosidase and also thiaminokinase (52) as a result of the development of resistance towards pyrithiamine. The most interesting feature of this resistant strain is the induced formation of an enzyme "pyrithiamine deaminase" which clearly indicates the mechanism by which the resistant strain could metabolise the antimetabolite (53). Besides these important observations it has been noted that the excretion pattern of several diffusible enzymes is also altered (54).

Biochemical studies on Vibrio cholerae

Research investigations in this laboratory (55-60) on the enzymes and metabolism of *Vibrio cholerae* have been centered around mainly on the identifica-

tion, characterization, purification and the determination of physicochemical properties of phospholipid splitting enzyme of *Vibrio* El Tor. The enzyme has been characterised as phospholipase B. Studies on the mechanism of formation of this enzyme in *Vibrio* El Tor have also been performed (61, 62).

Recently the induction of tryptophanase under the influence of various inhibitors of protein synthesis in different phases of growth of *Vibrio cholerae* has been studied (63). Informations on the constitutive β-galactosidase in *Vibrio cholarae* have also been presented (64). Further studies on the enzyme make up and other properties of normal and nitrofurantoin resistant strain of *Vibrio cholerae* are being actively carried out.

Studies on the active site of phosphoglucose isomerase

Information has been presented on the number and also nature of sulphydryl groups present in the enzyme phosphoglucose isomerase. The probable amino acids involved in the active site of the enzyme have been estimated with the help of photooxidation experiments. Detailed physicochemical properties of the enzyme have also been determined (65-68).

Biochemical studies on human blood cells under normal and leukaemic conditions

Reports have recently been made on the presence of several enzymes involved in uronic acid pathway and ascorbic acid metabolism in normal leucocytes, platelets and other blood cells and also in plasma and their alteration under leukaemic condition. Further studies have been made on the distribution pattern of several lysosomal enzymes in PMN granules and also in platelets under normal and leukaemic conditions. The results show interesting implications (69, 70).

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PROTEIN SYNTHESIS IN MITOCHONDRIA

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There are two ways in paying homage to a great scientist. One is to bring out the outstanding qualities of his head and heart and recount in personal reminiscence their influence in shaping the generation of scientists which followed him. The other is to dedicate to his memory a work which was initiated and developed at his inspiration. We prefer the second way for paying our homage to the great scientist.

It was always a passion with Guha to work on frontier areas and explore virgin fields. Protein biosynthesis is such a field where explosive developments have taken place during the last two decades. Here one can really see how vastly different disciplines and endeavours are brought to bear on the elucidation of a challenging problem and see "what part has been played by brilliant thought, skilful experimentations, new techniques and the capitalization on chance observation."

Protein syathesis is the most unique process in nature. Here a multitude of macromolecules participate and interact with one another in the orderly fashion of a bucket brigade. Here we have RNA molecules, the amino acid activating enzymes, a multiplicity of supernatant factors involved in chain initiation, chain propagation and chain termination, messenger RNA and different ribosomal components. To this system of complex macromolecules have been later added similar systems in mitochondria and chloroplasts, which are capable of independent synthesis of protein.

More than a decade ago almost simultaneously with a few other laboratories abroad we also demonstrated very clearly in higher plants that mitochondria are another satellite systems in the cells for protein biosynthesis. At that time we were studying the oxidation of glutamate by the plant mitochondria and the ^{14}C incorporation in respired carbon dioxide was far too less. To account for this anomaly we investigated ^{14}C uptake by other mitochondrial components. We were surprised to find that a major portion of ^{14}C has gone into proteins. At first we thought that this might be an artifact, involving contaminating effect of microsome which was at that time more well established as a site for the cellular synthesis of proteins, or coprecipitation, metabolic interconversion of the amino acid, formation of non-a-peptide covalent bonds, transpeptidation or other nonendergonic chemical reactions, or bacteria. All these possible factors were eliminated, as far as possible, by

very rigid experimentations. These mainly consisted of extensive washing of the mitochondria for the removal of adhering contaminants, characterization of the mitochondria and microsomes by chemical and physical methods, including electron microscopy, treatment of mitochondria with DNase to remove the traces of DNA on the mitochondria, demonstration of peptide bond formation by fluorodinitrobenzene reaction, ruling out the possibility of glutathione synthesis by transpeptidation reaction with subsequent attachment of the former to the protein through disulphide bond and ultimately demonstrating a net synthesis of protein.

The effect of several growth hormones on protein -synthesis by plant mitochondria and microsomes has also been investigated. Extensive use of metabolic inhibitors has been made for some in-depth study of the mechanism of protein synthesis by the plant cell organelles.

The work of protein synthesis has also been extended to other animal tissues and aberrant systems in malignant and transplanted fibrosarcoma tissues of rat.

These results have mostly been embodied on our publications (1-14). We next directed our efforts to the newer phase of the development of the problem.

Mitochondrion is a very intriguing biochemical problem of the day, having three major areas under intensive investigation : (1) membrane composition, assembly and function, (2) genetic studies and (3) synthesis of macromolecular components *viz.* proteins, nucleic acids, lipids, etc. Investigation in our laboratory falls under the third category.

Amino acid incorporation by mitochondria and mitoribosomes :

It is now well established that isolated mitochondria from divergent sources are capable of synthesizing protein (1-14). The over all mechanism for the *in vitro* synthesis of protein by the mitochondria from different sources appears to be more or less the same and has certain similarities with the bacterial ribosomal system.

Earlier we observed that the 105,000xg pellets of sonicated mitochondria, as isolated from the germinating seeds of *Vigna sinensis* (Linn.). Savi, were inactive with respect to amino acid incorporation but with the improved methodology and use of a better protecting agent, like dithiothreitol (DTT), whose use is only recently known, it has been possible to purify mitochondrial ribosomes and related enzyme systems which possess high incorporating activity (12a). We also extended the work on protein biosynthesis in malignant tissues, since cellular growth is often regulated *via.* qualitative or quantitative modifications of protein synthesis and cancer is also a problem of growth. A number of publications by us covers various aspects of cancer metabolism and only those related to protein biosynthesis are cited here.

An interesting finding by us is that the amino acid incorporating activity in both mitochondria and microsomes from malignant tissues is much higher than that found in the corresponding preparations from normal tissues. It is likely that under the stress of malignancy when construction of more structure materials, such as proteins, is needed for growth, the normal translation restraint is overcome to meet the higher demand for proteins. The nature of these proteins, synthesized by mitochondria or microsomes from malignant tissues is at present under our intensive investigation, since these proteins might have widespread effects in the total functioning of cells, leading to malignancy. Here we have at least two genetic system controlling a natural process of protein synthesis. There are other reasons for studying this aspect of the problem more intensively. We have demonstrated that mitochondria possess DNA of their own and are circular in nature. The isolated DNA was purified, rendered free of protein. The ultraviolet absorption spectra, the sedimentation velocity of this DNA and the action of deoxyribonuclease upon it, indicated that the compound was DNA and probably consists of one major component (3). This circular DNA is found in certain bacteria and viruses. The composition of the DNA of certain oncogenic viruses (adenovirus 12 and 18, papilloma and polyoma) show interesting similarities with those of mitochondria. So all these appear to be a very closely related process, a detailed study of which might be quite rewarding.

Study with synthetic messenger and "coding" problem :

Recent evidence has provided experimental support for the concept of a mRNA concerned with the transmission of information from DNA to protein. Our demonstration that synthetically prepared polyribonucleotides such as polyuridylic acid (poly U), polyadenylic acid (poly A) stimulate the incorporating activity of a protein synthesizing system indicates that such polymers act as messengers and "code" for the mitochondrial protein synthesis. Regarding the coding information for the mitochondrial protein synthesis, the opinion is divided. But it is true, at least, some of the mitochondrial RNA is transcribed on mitochondrial DNA. Our demonstration of the inhibition of synthesis of mitochondrial protein and RNA (13) by actinomycin D, mitomycin C, ethidium bromide and rifampicin supports the above hypothesis.

On the other hand, from the complementation experiments between mitochondrial RNA and nuclear DNA led some of the workers to conclude that there are two types of mitochondrial RNA one type being synthesized on nuclear DNA and the other on mitochondrial DNA (14). This type of experiments indicate that mitochondria obtain coding information for protein synthesis not only from mRNA transcribed on nuclear DNA but also from that of mitocondrial DNA. Different laboratories including that of ours have already

reported that externally added synthetic mRNA *viz.* poly U can permeate into intact mitochondria and stimulate there, specifically the incorporation of ^{14}C-labeled phenylalanine into proteins (15, 16). This observation may further support the hypothesis drawn from the complementation experiments, or in other words, if poly U can get entered into intact mitochondria, the permeation of nuclear mRNA into mitochondria within the cell is not impossible. In contrast, it has also been reported that protein synthesis by isolated mitochondria requires continuous synthesis of mitochondrial mRNA.

Effect of different inhibitors :

It is a very remarkable phenomenon that protein synthesis is susceptible to a surprisingly large number of natural inhibitors of diverse origin, structure and function. This indicates that protein biosynthesis is really the "Achilles heel" in cell metabolism. That is why a considerable insight into cellular process has been achieved from the study of the effect of different inhibitors on protein synthesis (13, 14).

Cycloheximide, a glutarimide antibiotic which inhibit microsomal protein synthesis has got no effect on mitochondrial protein synthesis in different systems. Chloramphenicol, a potent inhibitor of bacterial protein synthesis inhibits strongly the protein synthesis by isolated mitochondria. Emetine inhibits both mitochondrial and microsomal protein synthesis both in human normal and malignant tissues. The extent of inhibition is almost the same in both the cases of mitochondria and microsomes.

Fusidic acid, a potent inhibitor of bacterial translocation factor, has already been reported to inhibit mitochondrial protein synthesis in *Vigna sinensis* (11). In contrast, Kānzel reported that fusidic acid has got no effect on mitochondrial protein synthesis in *Nurospora crussa.* Although the overall mechanism of protein synthesis by the mitochondrial systems isolated from divergent sources is more or less the same, there may exist certain finer differences due to which they respond to a chemical substance in different ways. This may be further confirmed by the fact the effect of rifampicin on mitochondrial RNA synthesis in different systems, is quite different (17).

Effect of different growth promoters :

Kinetin, a plant growth hormone stimulates the *in vitro* protein synthesis in mitochondrial, isolated from 48-hr germinating seedlings of *Vigna sinensis* (Linn.) Savi. The effect appears at the early stage of incubation and attains the optimum at 10^{-5} concentration. Kinetin shows a synergestic effect with Indole-3-acetic acid in regard to amino acid incorporation (10).

Kinetin also stimulates *in vitro* mitochondrial protein synthesis in rat liver and hydrocortison, a growth hormone has got no effect on the process (18).

It has been shown that phenylalanine incorporation into proteins of mitochondria prepared from 48-hr germinating seeds of *Vigna sinensis* (Linn.) Savi can be stimulated by gibberellic acid (GA_3), kinetin and also polyuridylic acid (poly U) as a messenger. The incorporation is sensitive to a variety of inhibitors but some of their effect can be partly reversed by GA, and kinetin which indicates that these two plant hormones act at the level of messenger RNA which specifies the protein to be made or the protein synthesizing machinery involving the ribosomes and tRNA. Similar effect of the plant hormones has been observed in the incorporation of ^{14}C-labelled lysine directed by poly adenylic acid (poly A) (19). Further it has been shown that stimulation of protein synthesis in the plant mitochondria by 2,4-dichlorophenoxyacetic acid (2, 4-D) and kinetin results from the RNA synthesis sitmulated by these plant hormones (unpublished). All these results demonstrate very clearly a direct participation of plant hormones on protein synthesis and probably their sites of action.

Nature has a very uncanny way of covering up her tracks. This happens unquestionably with mitochondria. What little we have been able to uncover in respect to the mystery of mitochondrial protein synthesis during the last decade, we dedicate to the loving memory of our great teacher. Those who played a leading role in developing this work, particularly H. K. Das, S. K. Chatterjee, A. K. Banerjee, Sipra Banerjee, J. R. Bhattacharyya, N. C. Banerjee, B. Goswami, D. K. De, Aditi Sarkar and S. Ray and many others also associate themselves in this dedication.

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NEUROBIOCHEMISTRY AND DRUG ACTION IN BRAIN

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One of the major goals of biochemical neuropharmacology is to elucidate the mechanism by which the different neuropharmacologic agents alter the functional states of brain by interacting with the different structural and biochemical functional units of nerve tissue. With this broad perspective in view the neurobiochemical research programme in the Department of Biochemistry, Calcutta University, has been oriented, in the past two decades, in the following areas of neurochemistry and biochemical neuropharmacology.

1. *Effect of Anaesthetic agents and convulsants on the following Activities in Brain :*

Ever since it had been demonstrated nearly forty years ago by Questel and his school that barbiturate and other anaesthetic drugs affect metabolism of isolated braintissue and that there is a relationship between the hypnotic activity of anaesthetics and their abilities to suppression oxidative metabolism in brain *in vitro*. One major criticism of the results obtained under *in vitro* studies is that the concentrations of anaesthetics required to accomplish marked inhibitory effects on the metabolism of brain tissue are relatively large compared to the pharmacological dose needed to induce the unconscious state. Subsequent work carried out with potassium ion stimulated isolated brain cortex slices (J. J. Ghosh and J. H. Quastel, *Nature*, 174, 28, 1954) indicated that very low concentrations of barbiturates which are pharmacologically active also bring about marked inhibition of the respiratory processes in the brain. This inhibitory effect observed with potassium ion stimulated respiration is found in slices and does not occur in homogenate or mince, indicating that this is linked with the integrity of brain cell membranes.

In view of the well-known sensitivity of brain tissue to ammonia, several studies have been carried out on the control and regulation of ammonia forming and ammonia removing mechanisms in normal and drug treated brains (S. R. Guha and J. J. Ghosh, *Ann. Biochem. Exp. Med. Biochem.*, **19**, 163, 1959 ; S. R. Guha and J. J. Ghosh, *Ann. Biochem. Exp. Med.*, **19**, 255, 1959). The

glutamine transaminase enzyme, which has been characterized and found to be present in mammalian brain (S. R. Guha and J. J. Ghosh, *Ann. Biochem. Exp. Med.*, **19**, 33, 1959) may play an additionally important role in the regulation of ammonia metabolism in brain.

2. *Brain Ribonucleoprotein in Normal and Neurotropic Drug Treated Conditions :*

For a number of years, the work of this laboratory has been focussed on the action of different convulsant and hellucinogenic drugs at the level of ribonucleoprotein constituents of brain tissue, studied biochemically and histochemically. Most of the cytoplasmic ribonucleoproteins of nerve tissue is located in the Nissl bodies, although nuclei and nucleolar regions of nerve cells contain a significant amount of nucleoprotein. The characteristic histological picture of the distribution pattern of ribonucleoprotein particles showed marked changes in the form of chromatolysis in brain tissue selection from picrotoxin, strychnine, tetanus toxia and mescaline treated rats (K. Sikdar and J. J. Ghosh, *J. Neurochem.*, **11**, 545, 1964, K. Sikdar and J. J. Ghosh, *J. Neurochem.*, **13**, 205, 1966). In order to characterize the mode of action of different psychotropic drugs at the level of brain cortex ribonucleoproteins, detailed studies have been done on the isolation, characterization (in terms of chemical composition, UV absorption characteristics, sedimentation, Molecular weight data etc.) stability properties and secondary structures (in terms of hydrogen bonded structures of ribosomal RNA component) of rat brain cortex ribosomes. The rat brain cortex ribosomes show a major component with sedimentation coefficient of 82.5 at 20°C and infinite dilutions in sucrose-KCl-Mg cacodylate buffer, intrinsic visconty, 0.06 dl/g and molecular weight about $4.9X10^6 \pm 15\%$. The RNA component of the ribosomes of normal adult rat brain cortex has also been characterized and have been shown to consist of two nearly homogeneous components *viz.* 28.45 and 18.25 (at infinite dilutions and 20°C). The nucleotide composition of the ribosomal RNA from brain cortex tissue revealed that guanylic and cytidylic acids were highly predominating. Stability studies of isolated brain cortex ribosomes revealed that the presence of Ca^{++} or Mg^{++}, phosphate ions or polyamines determine to a great extent the integrity of brain cortex ribosomal structure. (R. K. Datta and J. J. Ghosh, *J. Neurochem.*, **10**, 363, 1963 ; R. K. Datta and J. J. Ghosh, *J. Neurochem*, **11**, 595, 1964 ; R. K. Datta, S. Sen and J. J. Ghosh, *Biochem. J.*, **144**, 847, 1969 ; J. J. Ghosh, *Proc. Int. Soc. Neurochemistry* (1967), **2**, 57).

Results emerging from the studies on the molecular mode of action of convulsant drugs like picrotoxin and strychnine and hallucigenic drugs like mescaline indicate that the structural integrity and functional properties of ribo-

somes, isolated from the brain cortex tissues of drug treated rats, are affected (J. J. Ghosh, R. K. Datta and K. C. Bhattacharyya, *Canad. J. Biochem.*, **43**, 959, 1965 ; R. K. Datta and J. J. Ghosh, *J. Pharmacol. Exp. Therap.*, **150**, 449, 1965 ; J. J. Ghosh, *J. Ind. Chem. Soc.*, **44**, 872, 1967). While making a detailed study on the mode of action of mescaline at the level of brain cortex ribosomes, it has been shown that mescaline action causes significant decrease in the hydrogen bounded structure of the 28 S RNA component of the total ribosomal RNA species (R. K. Datta and J. J. Ghosh, *Biochem., J.* **117**, 961, 1970 ; R. K. Datta and J. J. Ghosh, *Biochem. J.*, **125**, -13, 1971). It has further been observed that ribosomes, isolated from mescaline treated brain tissue, have decreased amino acyl-t-RNA binding capacity with concomitant decrease in the overall protein synthesizing ability (R. K. Datta and J. J. Ghosh, *Brain Res.*, **33**, 193, 1971 ; R. K. Datta, W. Antopol and J. J. Ghosh, *Naunyn- Schmiedeberg's Arch. Pharmacol.* (*Springer Verlag*), **277**, 319, 1973).

3. *Psychotorpic Drug Action at the level of Microsomal and Synaptosomal Membrane Bound Enzymes of Brain :*

Although much evidence has accumulated about the role of membrane lipids in drug action, remarkably little precise information is available about the interaction of neuropharmacologic drugs with neuronal membrane components, particularly at the levels of microsomes and synaptosomes, which control the storage, transport and release of cations, amino acids and biogenic amines. Drug like strychnine (B. K. Pal and J. J. Ghosh, *J. Neurochem.*, **15**, 1243, 1968), morphine (S. K. Ghosh and J. J. Ghosh, *J. Neurochem.*, **15**, 1975, 1968), imipramine (D. Nag and J. J. Ghosh, **4**. *Neurochem.*, **20**, 1021, 1973) and delta-9-tetrahydrocannabinol, the major active component of cannabis, (M. K. Poddar and J. J. Ghosh, United Nations Document, ST/SOA/SER S/36, 1972) have been shown to affect characteristically the activities of Na--K-ATPase, acetyl choline esterase and glutamine synthetase in synaptosomal and microsomal membranes of brain tissue.

From foregoing studies in the field of biochemical neuropharmacology, it is evident that neuropharmacologic drugs may affect the brain function by acting at the level of important biochemical parameters of brain, the detailed studies of which are included in the ongoing research programme in neurobiochemistry.

STRUCTURES OF GLYCOLIPID (ANTIGEN) ON SHEEP AND GOAT ERYTHROCYTES

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The glycolipid present on the surface of sheep erythrocytes is commonly known as Forssman hapten. Forssmann in 1911[1] detected the presence of this Forssman hapten like substance in the kidney of guinea pig. Since then the various workers[2] tried to isolate the Forssman hapten in pure state either from sheep erythrocytes or from the kidney of horse and other animals. Yamakawa *et al*[3] and aslo Makita *et al*[4] were able to isolate a compound and arrived at its structure to be α-N-acetyl-D-galactosamine-O-β-D-galactose-O-β-D-galatose-O-β-D-glucose-ceramide. Recently Dasgupta and Ghosh[5] have isolated and purified the Forssman hapten from sheep erythrocytes. The constituents of the hapten was identified to be galactosamine, galactose, glucose and ceramide. The structure of the hapten has been studied by stepwise enzymatic degradation with specific enzymes and the estimation of the product liberated at each step. Immunological reactions of the hapten were studied by the inhibition of agglutination and hemolysis in presence of guinea pig complement.

Anti-A serum is found to agglutinate sheep erythrocytes in a manner analogous to that observed with human A cells. Further, the Forssman hapten isolated and purified was capable of inhibiting both A cells and anti-A serum agglutination as well as sheep cells and anti-A serum agglutination. The phenomenon in preventing agglutination by the hapten suggested that in the Forssman hapten the terminal group present must be alike to that present in A group substances. The terminal group of A substances is known to be N-acetyl-D-galactosamine. Further N-acetyl-D-galactosamine can inhibit agglutination between A cells and anti-A serum. Similar inhibition by N-acetyl-D-galactosamine was observed with sheep cells and anti-A serum. This indicated the presence of N-acetyl-D-galactosamine to be the first terminal group of Forssman hapten.

The first terminal group of the hapten was confirmed by enzymatic hydrolysia with specific enzyme, α-N-acetyl-D-galactosaminidase which was isolated and purified from the hapatopancreas of *Achatina Fulica*. This enzyme completely removed-α-N-acetyl-D-galacosamine. The liberated galactosamine was estimated quantitatively by the method of Elson and Morgan[6].

As the second terminal group of A substances is β-D-galactose, it is natural to search for the second group of the hapten to be β-D-galactose. The residue left after the hydrolysis of *Achatina* enzyme was subjected to enzymatic hydrolysis with β-D-galactosidase of rat intestine according to the method of Brady *et al*[7]. The liberated sugar was identified to be β-D-galactose. Thus the first two terminal groups of the hapten are same as those present in A group substances namely, α-N-acetyl-D-galactosamine-O-β-D-galactose. The glycolipid isolated by Koscielek *et al*[8] from human A cells was identified to have the structure as α-N-acetyl-D-galactosamine-O-β-D-galactose-O-β-N-acetyl-D-glucosamine-O-β-D-galactose-O-β-D-glucose-ceramide.

The residue left after the removal of the first two groups from the hapten was soon identified to be β-D-galactose-O-β-D-glucose-ceramide or cytolipin H. This was confirmed both from the R_f on the thin layer chromatography and also by the two stage enzymatic hydrolysis with rat brain enzymes according to Rapport *et al*[9]. Thus the structure of the Forssman hapten of sheep erythrocytes established as α-N-acetyl-D-galactosamine-O-β-D-galactose-O-β-D-galactose-O-β-D-glucose-ceramide and this is in agreement with the structure of the Forssman hapten as put forward by Makita *et al*[4] who isolated it from equine kidney and spleen.

Since sheep and goat belong to the same species of animals, it is likely that the Forssman hapten should be present in goat erythrocytes. Further, the cytolipin K isolated from goat kidneys according to the method of Rapport *et al*[10] showed almost identical in structure to that of Forssman hapten. The immunological behaviours of goat cells were however different and did not agglutinate in presence of anti-A serum as observed with sheep cells. Further, goat cells cannot be sensitized with hemolysin (anti-sheep cells) and hindered hemolysis in presence of complement. The same was true with sheep cells when sensitized with anti-goat cells serum. It became evident that the glycolipid or antigen on the surface of goat red cells is different from that present in sheep red cells.

The goat cells were found to be agglutinated by anti-B serum but the reaction is not as sharp as that observed with B cells. The glycolipid isolated from goat erythrocytes by chloroform-methanol (3 : 1) extraction followed by silicic acid column inhibited the agglutination reaction between B cells and anti-B serum. Thus the terminal group of goat glycolipid was identified to be β-D-galactose. This was confirmed by inhibition of agglutination reaction in presence of D-galactose for B cells and anti-B serum. In this inhibition test the degree of inhibition is comparable to that observed with goat cells and anti-B serum.

The goat glycolipid on acid hydrolysis yielded glucosamine, galactose, glucose and ceramide. It gave a different R_f value from that of the Forssman hapten on thin layer chromatography.

The terminal group of goat glycolipid was suspected to be -D-galactose as it inhibited agglutination between B cells and anti-B serum. To confirm that D-galactose is the terminal group of goat glycolipid it was treated with the enzyme β-D-galacosidase of rat intestine[9] and the released galactose was estimated. The second terminal group was identified, to be β-N-acetyl-D-glycosamine when the material left after β-D-galctosidase treatment was found to be acted on preferentially by the enzyme, β-N-acetyl-D-glucosaminidase of goat testes[11]. The residue left after two successive treatments of the above mentioned enzymes showed to be β-D-galactose-O-β-D-glucose-ceramide as its R_f value was identical to cytolipin H on thin layer chromatography. The complete structure of goat glycolipid comes to β-D-galactose-(1—4)-O-β-N-acetyl-D-glucosamine-(1—4)-O-β-D-galctose-(1—4)-O-β-D-glucose-ceramide and is different from that of sheep glycolipid or Forssman hapten.

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PRODUCTION OF PROTEIN BY SUBMERGED GROWTH OF EDIBLE MUSHROOMS

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Introduction

India and elsewhere researches are in progress to make use of many "Unconventional" sources like soyabean and other legumes, oil seed cakes, dried fish meal product, leaves and grasses and microorganisms, as much wanted food supplement. Out of these, microorganisms appear to have distinct but not yet fully realized potentialities as future source of energy and protein for under-fed people and as regenerative source of food in manned space vehicles and station. The suitability of microorganisms as food material based on three advantageous factors (*i*) possibility of utilisation waste materials for cell growth by microorganisms resulting the reduction in environmental pollution (*ii*) rapid rate of proliferation of microbial cells and (*iii*) favourable economics of the fermentation technology which has undergone tremendous development during last twenty years in terms of time labour and space.

Microorganisms which may be introduced in human diet include algae (fresh water and marine), yeasts, bacteria and molds. Some of these are already being consumed by different sections of world population.

Mushrooms as food

Edible fungi of higher order have for long been greatly esteemed as food. The fleshy fungi, particularly mushrooms have received wide popularity due to their flavour as well as high protein, vitamins and mineral contents. Some of the most popular edible species are common mushroom, *Agaricus campestris*; the shaggy mane, *Coprinus comatus*; the common ink cap, *C. atramentaris*; the glistering ink cap, *C. micaceus*; the oyster mushroom, *Pleurotus ostreatus*; the parasol mushroom, *Lepiota procera*; the honey agaric, *Armillaria mellea*; the velvet–stemmed mushroom, *Collybia Velutipes*; the morel, *Morchella esculenta*; Coral fungi and puffballs. Most of these mushrooms grow in wild and collected from their natural habitat. Planned cultivation of some selected popular species are practised to get regular supply of mushroom fruit bodies of uniform quality at large scale.

Production of mushroom mycella by submerged culture

The idea of development of submerged culture methods for the production of mushroom mycelia was stimulated by the success of deep tank fermentation technique in the antibiotic industry. It was Humfeld (1) who first showed that mycelial growth of certain strain of mushrooms could be achieved by this method of cultivation. The method offered low cost production of mycelium having high nutritive value and acceptable flavour using easily available substrates. It was soon realised that by similar process mycelia of many other edible species of higher fungi could be produced economically (2-8). However, it was found that not all fungi of this class could be cultivated *in vitro* (5), some of these which may be grown on solid media *in vitro* failed to grow under submerged condition. A comprehensive list of species which could be grown in submerge culture is compiled by Worgan (5). This list includes about 90 edible fungi out of which *A. campestris, Tricholoma nudum, M. esculenta, M. crassipes* and *M. hortensis,* are among the few which have been studied in detail.

Production of protein by *A. Campestris* S_{12} in submerged fermentor

Selection ot the strain

Fruit bodies of *A. campestris* were collected from different localities around Calcutta and their adaptability in submerged cultivation were tested in potato-dextrose broth. The ability to grow in liquid-shake culture was found to be restricted in a relatively small number of strains, out of 18 strains tested, only 6 could adapt to submerged propagation. The protein content of mycelia obtained in submerged codition is also different in different strains. The strain selected for detail studies (*A. campestris* S_{12}) yielded mycelia contains about 42% protein on dry weight basis. Mycelia were non-toxic to mice.

Selection of media : Carbon, nitrogen and mineral nutrition of A. campestris S_{12} :

The production and protein content *A. campestris* S_{12} mycelia under submerged condition are dependent on medium nitrogen and carbon sources. The efficiency of utilisation of various nitrogenous compounds as calculated from the amount of nitrogen recovered in mycelial material is dependent on the nature of nitrogen compounds provided in the medium. Inorganic nitrogen compounds, such as ammonium nitrate, ammonium sulphate, ammonium phosphate and sodium nitrate ; or urea poorly support growth of the present strain. Complex organic nitrogen compounds are growth stimulatory. Peptone is the best supporter of growth, the most protein-rich (50% on dry weight basis) mycelium could be obtained with casein hydrolysate, whereas, the maximum recovery of nitrogen from the medium could be obtained with skimmed milk powder (9). Among different carbon compounds tested, mannitol appears

to be the best in consideration of extent of utilisation, efficiency of recovery of carbon in cell material and protein content of mycelia produced. Economic coefficient, which is an index of utilisation of carbon compounds for building up of cellular material is high for xylose and mannitol. These values for glucose and fructose are comparable and are little lower than those for xylose and mannitol. Disaccharides are utilised at slow rate. Galactose, rhamnose, raffinose, sodium acetate and sodium citrate are not utilised (10).

The requirements of *A. campestris* S_{12} for trace elements for growth were tested in chemically defined medium (11). The optimum concentrations of Fe^{++}, Zn^{++}, Mn^{++}, Cu^{+} and Ni^{++} are 0.125, 0.5, 0.5, 0.05 and 0.025 mg per litre respectively. All these metal ions are growth inhibitory to different degrees at higher concentrations. Co^{++} is growth inhibitory even at very low concentration. The protein content of mycelium is also influenced by trace elements. The concentrations of metal ions which give maximum protein per gram of dried mycelium are not same as those necessary for maximum growth. Further more, Cu^{++} and Ni^{++} are needed for growth but inhibitory for protein accumulation in mycelia. So trace element concentration of cultivation medium should be critically balanced in order to obtain maximum protein harvest (11).

Physical and chemical changes during fermentation

The growth of *A. campestris* S_{12} in mannitol- peptone- yeast extract broth is a slow process. There is a time lag of 1 day for the onset of the growth and the maximum supportable growth attains after 7 days. The entire 9 day growth period of the organism could be divided into 4 arbitrarily defined phases based on the rates of utilisation of sugar, nitrogen compounds, inorganic phosphorus and rate of change of pH of the medium. Protein content of mycelial material harvested at different growth phases are variable. The maximum protein could be harvested after 144 hours and before 168 hours of the fermentation (12).

Effect of plant growth hormones on protein production

Indoleactic acid and kinetin stimulate the growth of *A. campestris* S_{12} at optimum concentration of 0.4 mg/litre and 0.75 mg/litre respectively. Both of these plant hormones are growth inhibitory at concentration above the optimum one. The protein content of the mycelia is increased only to a small extent in hormone treated culture. The stimulation is also dependent on the concentration of the hormones. The concentration of either kinetin or indoleacetic acid which is maximum stimulatory for the growth of the fungus may not stimulate protein accumulation in the mycelium to the maximum extent, on the reverse, the concentrations of the hormones needed for the maximum protein accumulation in the mycelium are growth inhibitory. So overall benefit

of incorporation of these two plant growth hormones in the culture emdia for the protein production by *A. campestris* S_{13} would be very small (Guha, A. K. and A. B. Banerjee, *communicated for publication*).

Nutritive value of mycelia and mycelial protein obtained from submerged fermentation

The mycelium of *A. campestris* S_{13} harvested from submerge culture is a rich source of riboflavin, niacin, iron and magnesium but its thiamine and calcium content is low. Vitamin content of mycelium is dependent on culture age and reaches maximum on 7th day of fermentation. The fibre content of dried mycelia varied between 4.9-10.8%, the mycelia from older culture have higher fibre content (13). Protein isolated from the mycelia contains almost all amino acid essential for human nutrition. It is particularly rich in leucines and lysine, but poor in sulphur amino acids and valine. Chemicals core, biological score and essential amino acid index for the purified protein are 25, 33.8 and 58.4 respectively (13).

Conclusion

It is well recognized that diets consumed by the low income groups in developing countries like India do not provide adequate amounts of protein of high nutritive value, and that consequently protein malnutrition is widely prevalent. The problem of utilisation of available additional sources of proteins for overcoming protein deficiency in the diets of Indian people has engaged the attention of food scientists of this country during the last quarter century. The present study was undertaken to develop a suitable strain of *A. campestris* for submerged culture. The fermentation conditions of the strain so developed for maximum protein production were standardised.

The selection of the organism, *A. campestris* is based mainly on two reasons. (*i*) A good amount of technical data is available on the submerged cultivation of this organism. The most the work in this respect was carried out by Humfeld and Shugihara (14), the pioneers of this field. Unfortunately in India no attempt is made so far to make use of this potentially useful source of protein, (*ii*) At least a section of Indian population is familiar with the taste and nutritive value of this fungus.

The most important factor determining the biological value of a protein is the relative concentration of amino acid, particularly of the essential ones, present in it. Protein of *A. campestris* S_{13} is rich in lysine and leucines, the concentrations of these amino acids exceed that of reference protein recommended by FAO. The essential amino acid make up of *A. campestris* S_{13} protein compares favourable with those of proteins from different other micro-

organisms. It has almost similar quantitative distribution of essential amino acids as that of *S. cerevisiae*.

It is apparent from the *in vitro* assessment figures that *A. campestris* S_{12} protein has low biological value in comparison to proteins of animal origin. This low biological value is due to low sulphur amino acids and valine contents. So this protein would not be suitable in animal nutrition if used alone. However this protein may be used for supplementation and fortification of cerael diets. The cereal proteins used by Indian population are generally deficient in lysine, tryptophan, threonine and methionine. Some of these deficiencies could be balanced by by fortification of diets with *A. campestris* S_{12} protein. There is plentiful evidence in the literature to show that two or more proteins of inferior growth promoting values can be so blended that their amino acid deficiencies are mutually made up with the result that the mixture contains proteins of superior nutritive value.

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NATURAL ANTITHIAMINES

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According to Mellanby (1) the classical concept of vitamin deficiencies was modified by new findings that besides the absence of vitamins, the symptoms of vitamin deficiency were also shown by presence of certain natural toxic agents (2). Kodicek (3) emphasized the term 'toxamin' to cover the structural analogues as well as other vitamin-inactivating agents. Later on, the structural analogues were designated as vitamin antagonists (4) whereas the naturally-occurring vitamin-inactivating agents were termed as anti-vitamins (5).

Of the water-soluble B-vitamins, thaimine is present in several foodstuffs which also contain some thiamine-inactivating agents (6). These are generally termed as antithiamines have been classified into two types which are mentioned below :

Type I : Antithiamine of large molecule

Type II : Antithiamine of small molecule

Antithiamine of large molecule :

Spitzer *et al.* (7) in 1941 observed a condition in chick similar to thiamine deficiency by feeding raw fish and that incubation of thiamine with raw carp fish intestine for fifteen minutes resulted in 'a loss of 50-100% of the biological activity of thiamine. Evan (6) demonstrated the so-called 'Chastek Paralysis' of foxes by feeding 10% or more of fresh whole fish in the diet could be prevented by administration of 10 mg of thiamine per day. The enzymatic nature of the factor was confirmed by Sealock and Goodland (8, 9) who also studied the destructive nature of this enzyme on thiamine and designed it as 'thiaminase.'

This enzyme was also found to be present in the Atlantic herring (*Cluper harrengus*) and in clams, shrimgs and some mussels. Recently thiaminase was also detected in bacterial system (10). Two types of thiaminase are known according to their mode of action such as (*a*) Thiaminase I and (*b*) Thiaminase II.

The occurrence of thiaminase I is wide among the various organisms such as *Bacillus thiaminolyticus, Clostridium thiaminolyticus* etc. Fresh water fish, shell fish as well as bracken fern are also good sources of thiaminase I. Airth *et al.* (11) purified thiaminase I from the bacterial source, *Bacillus thiamino-*

lyticus M in the free state. It is a single protein with an absorption maximum at 277μ and minimum at 252μ. Various physicochemical properties of thiaminase I as ultracentrifugation, polyacrylamide gel electrophoresis and immunediffusion in agar-gel demonstrated a molecular weight of 44,000. Ebata and Murata (12) reported a molecular weight of 40,000 calculated from the sedimentation velocity coefficient and diffusion coefficient of thiaminase I isolated from other bacteria. According to Wittliff and Sirth (13) thiaminase I catalysed the decomposition of thiamine by a base exchange reaction involving displacement on the methylene bridge of the pyrimidine moiety. Thiaminase I activity is generally determined by a modified method of Wittliff and Sirth (11) where aniline is used as base. The product as aniline derivative is measured spectrophotometrically by an increase of optical density at 248μ.

Thiaminase I is active against thiamine, thiamine pyrophosphate and other thiamine derivatives with 4-amino group of pyrimidine moiety. The enzyme isolated from different sources is activated by a number of aromatic amines, heterocyclic amines and sulfahydryl compounds. Several divalent cations such as Fe^{++}, Cu^{++}, Mn^{++} have some inhibitory role on thiaminase. Both p-chloromercurobenzoate and monoiodoacetic acid have some potent inhibitory role on the bacterial thiaminase I. The optimum pH of thiaminase I (14) varies from source to source. The bacterial thiaminase I has a broad pH optimum of 5.8 to 6.8. Similarly, that of certain shell fish (*Meretrix meretrix*) has a pH optimum of 5.0. The enzyme isolated from bracken fern has a pH optimum of 7.8. It is more or less heat stable enzyme and it is reversively inactivated at temperatures above 45°C but not exceeding 65°C. The bacterial thiaminase I retains most of its activity at 60°C even after 20 minutes in a phosphate buffer of pH 5.8.

(*b*) Thiaminase II :

It is present mainly in the bacterial system such as *Bacillus aneurinolyticus*, fungi, *Micrococcus pyrogenes, E. coli, Candida aneurinolytica* etc. (15). Thiaminase II has been successfully isolated from *Bacillus aneuronolyticus* using Ikehata's synthetic medium (16). It is an extracellular enzyme and has been crystallised by Ikehata (16). Ultraviolet absorption spectrum (17) indicates a simple protein with the absorption maximum at 276μ and a minimum at 252μ. Various physicochemical properties of the enzyme demonstrate its molecular weight of 1,00,000 (16). Basically it is a hydrolysing enzyme to split thiamine into two moieties without the participation of a base (18). The enzyme activity is measured (19) after incubation of thiamine, buffer, and enzyme followed by the determination of residual thiamine by thiochrome method.

Besides thiamine, thiaminase II (15) has affinity to hydrolysis thiamine derivatives with the side chain intact at 5-position of the thiozole moiety. It has no effect on thiamine pyrophosphate. Cysteine, EDTA etc. (17) act as an activator of the enzyme. Various aromatic amines, heterocyclic amines, heavy metal ions such as Fe^{++}, Cu^{++} and Zn^{++} markedly function as inhibitors of the enzyme. Thiaminase II has a broad pH optimum dependent on the sources. Thiaminase II from *O. lactis, C. aneurinolytica* has an optimum pH of 7.0 whereas that isolated from *B. aneurinolyticus* is 8.6. It is more or less a heat stable enzyme and it has optimum activation temperature of 60°C.

Antithiamine of small molecule

Thiamine deficiencies symptoms developed in swine (20), pigeons and rats (21) by feeding exclusively the rice milling products and later on similar phenomena were described by McCollum, Simmond and Pitz (22) as well as Hart *et al.* (23) in swine using wheat germ and other wheat products. Bhagat *et al.* (24) also confirmed the presence of thiamine-inactiving factors in cereals under *in vitro* conditions as determined by thiochrome method. These workers (*loc. cit*) also reported the presence of heat-stable thiamine-inactivating agents in ragi (*Elusine coracana*), bajra (*Pennioetcem typhoidecem*), green gram (*Phaseolus radiatus*), mustard seed (*Brassica juncea*), cotton seed (*Bombex malabericum*) etc.

Recently Somogyi *et al.* (25-28) have successfully isolated and characterised a heat stable antithiamine factor commonly known as caffeic acid or 3,4 dihydroxy cinnamic acid. The existence of this acid in different foodstuffs as blue berries (*Vaccinium myrotillus* L), fern (*Pferidicem aquilincem*) have also been demonstrated by them (27). They (29) also claimed that the reaction mechanism between thiamine and the isolated antithiamine *i.e.* caffeic acid was found to consist of two phases as distinguished by their marked differences in reaction rate, reversibility as well as dependency on temperature, oxygen and pH. They further observed (*lac cit*) the interaction between thiamine and caffeic acid at the initial stage is a reversible phenomenon with high reaction constant and independent of temperature and pH. At the second phase thiamine-inactivation is an irreversible phenomenon as well as dependent on oxygen, pH and temperature.

Somogyi (30) further determined the antithiamine activity of several phenolic compounds and suggested that the phenolic compounds with two hydroxyl groups at their ortho positions with respect to para aliphatic chain of the aromatic ring have been shown to have maximum antithiamine activity. Compounds with substitution at the para aliphatic chain by nitrate, aldehyde, or hydroxyl have no antithiamine activity. Again change of hydroxyl group from

ortho to para position decreased the antithiamine activity whereas with the change of hydroxyl group from ortho to meta position, the antithiamine activity was negligible.

Recently two new antithiamines have been isolated in the pure state from two different sources in this laboratory. Chaudhuri (31) working on the isolation of the antithiamine factor present in rice bran was able to purify it appreciably. Following this work, De and Chaudhuri (32) have succeeded in isolating this factor in the pure state and characterised it to be a glucoside. Its glucose content has been measured after hydrolysis and found to be about 14% on weight basis. On hydrolysis with dilute mineral acid, an oily layer separates out which contains two fractions as demonstrated by thin layer chromatography. These two fractions have subsequently been separated out and the first elute has been designated as "Fraction A" and the second elute as "Fraction B." The first elute "Fraction A"—yellow viscous oily substance which has been found to be homogenous by thin layer chromatography, retains all the antithiamine activity and the second fraction B.—pinkcoloured compound has been shown to have no antithiamine activity at all.

Various physicochemical properties such as ultraviolet spectrum analysis of "Fraction A" show absorption maximum at 210 μ and minimum at 280 μ which are characteristic of simple aromatic chromophore. The spectrum in the alkaline medium showing a slight shift of the maximum to 215 μ and minimum to 285 μ indicates the presence of ionisiable group (s) in the molecule. Infra-red spectrum of "Fraction A" demonstrates the presence of strong-bonded hydroxyl group in the molecule. This, in conjunction with the strong band at 1695 cm^{-1} indicates the presence of unsaturated carboxyl acid function in the molecule. Both UV and IR spectra along indicate that "Fraction A"—is a ortho dihydrony aromatic compound with Mass spectra data having a aliphatic side chain.

Microbiological studies (33) using *S. aureus* and enzymological studies using erythrocyte transketolase system demonstrate that the isolated antithiamine (glucoside) and its hydrolysed product—"Fraction A" could inactivate both thiamine and thiamine pyrophosphate under *in vitro* conditions. The mechanism of inactivation is under study.

Following the observation of Bhagat and Devi (*loc. cit*) Chakravarti and Chaudhuri (34) attempted to purify the antithiamine factor from mustard seed (*Brassica juncea*) and were able to obtain it in a highly purified state. Subsequently Bhattacharya and Chaudhuri (35) working on this problem isolated this antithiamine factor from mustard seed (*Brassica juncea*) in the pure state as light-yellow needle-shaped crystalline compound having M.P. of 92°C.

The microanalysis and mass spectrum shows the molecular formula of $C_{13}H_{14}O_5$ having molecular ion at m/e 238. The infra red and ultraviolet spectral analysis indicates the presence of aromatic chromophore with conjugated carbonyl (1690 and 1640 cm^{-1}) and phenolic hydroxyl groups.

The structure of the compound could be deducted from the NMR spectrum (60 MHz, $CDCl_3$) which showed sinals for a disubstituted conjugated *trans* double bond (AB quartet, δ_A 7.63, δ_B 6.30, J_{AB} 16 Hz), two equivalent aromatic protons (2 proton signglets at 6.80) and three OMe groups (6 proton singlets at δ6.90 and 3 proton singlets at δ3.81). All these indicate that the compound is the methylester of sinapic acid (3,5-dimethoxyl-4-hydroxyl cinnamic acid).

In conclusion it may be emphasized that the heat-stable antithiamines such as caffeic acid from fern etc., glucoside and "Fraction A" from rice bran as well as methylester of sinapic acid from mustard seed initiate a new chapter to study the interaction between the antithiamine and thiamine or thiamine pyrophosphate under *in vitro* as well as *in vivo* conditions. The existence of heat-stable antithiamines in different foodstuffs along with thiamine initiates an interesting line of research in the field of nutritional biochemistry.

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EVOLUTION, BIOSYNTHETIC ABILITY AND NEED FOR VITAMIN C

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It was a memorable event in my life when I met Professor B. C. Guha on a day in November, 1953. At the very first sight my intuition told me that he was 'The Master' whom I had been searching so long. Not only he gave me an opportunity to carry on researches with him, but for long eight years of association he also preached me how to be a scientist. "If you want to be scientist," he used to say, "you must dream in Science." "Research is truth seeking and honesty and integrity should be the basic qualification of a scientist," he preached, "you must be imaginative but you should have clear objective," "You must be pregnant with ideas, but at the same time you should execute the experiments in finest details." He used to caution, "you must be ready to surrender your hypothesis at any time if the facts are adverse to it." When we showed (1) that Albert Lehninger (2) was wrong in saying 3-keto-L-gulonic acid as an intermediate in the biosynthetic pathway of ascorbic acid in the rat Professor Guha at once said, "The duty of a scientist is not only to show which is wrong, but he must also find out what is right." This impetus led us to identify 2-keto-L-gulonolatcone as the penultimate precursor of ascorbic acid in the rat (3). Professor Guha's work on the relationship between vitamin C synthesis and phylogeny and his discovery that besides man, monkey and the guinea pig, two more species, namely the fruit bat and the red vented bulbul are also incapable of synthesizing ascorbic acid (4) is in the word of C. G. King (5) "A new and very interesting landmark to the history of vitamin C." Undoubtedly, many more landmarks would have been added but for the immature death of the doyen of Indian Biochemists. Following in his footsteps, further work has been carried out and the result described below represents an extension and projection of late Professor Guha's work.

EVOLUTION AND THE BIOSYNTHESIS OF VITAMIN C

Evolution is an extremely complicated multiphased process acting at all levels in the protoplasmic hierarchy and it produces at intervals organized systems whose survival is determined on adaptation. The interaction of mutation, genetic drift and natural selection and the taking on of new adaptive efficiency play an exceedingly important part. The acquisition of greater adaptive efficiency by new populations is sometimes accompanied by loss of functions. When the environment makes up for the lost biochemical function by furnishing

the product, a loss of function does not become detrimental and the species continue to survive. One such example is the loss of capacity to synthesize ascorbic acid (vitamin C) in course of evolution. The biosynthetic capacity started in the kidney of amphibians, resided in the kidney of reptiles, transferred to the liver of mammals and finally disappeared from the guinea pig, the flying mammals, monkeys, apes and man (6-11). A similar transition in the biosynthetic ability was observed in the branched evolution of birds. The primitive birds synthesize ascorbic acid in the kidney. The more evolved passeriformes produce it in the liver while a number of other highly evolved passers are incapable of synthesizing the vitamin (12). Recently it has been shown that insects, invertebrates and fishes are also incapable of synthesizing ascorbic acid (13, 14). Figure 1 shows how the overall pattern of ascorbic acid synthesis by different species of animals is correlated to their phylogeny.

Evolution of the biosynthetic capacity : The incapability of insects, invertebrates and fishes to synthesize ascorbic acid would apparently raise the question whether ascorbic acid is an essential requirement for these species. Of course, it has been reported that salmon, trout (15) and the desert locusts (16) are dependent on dietary ascorbic acid. But their need may be small so that it is satisfied in their food. Apparently, the biosynthetic mechanism did not evolve in these earlier species of the phylogenetic tree.

Whereas the fishes are unable to synthesize the vitamin, kidney homogenate from the water living tadpole synthesized a significant amount of ascorbic acid *in vitro* (220-250 μg/mg kidney/hour). The emergence of the biosynthetic ability in the amphibians would suggest that a greater need of the vitamin was somehow linked with the evolution of vertebrates from aquatic to the terrestrial environment. The evolution of all lines of vertebrates generally required loss of specialization as new specialization came up. The evolution of ammonotelic fishes to the ureotelic amphibians is thus associated with the newly specialized capacity to synthesize ascorbic acid.

When the descendants of certain Crossopterygian fishes ventured out of the water and crawled upon the mud banks of streams and lakes at the end of Devonian period to become the first amphibians (17), the vertebrates entered a completely new course of evolutionary development. The step from the aquatic to the terrestrial mode of life was a profound change involving a tremendous range of adaptations under strong selection pressure. The primitive quadrupedal vertebrates had to face a variety of extreme stressful conditions like supporting of body weight, locomotion on land against gravity, high oxygen tension, desiccation by dry air and hot sun. Perhaps, the requirement of as-

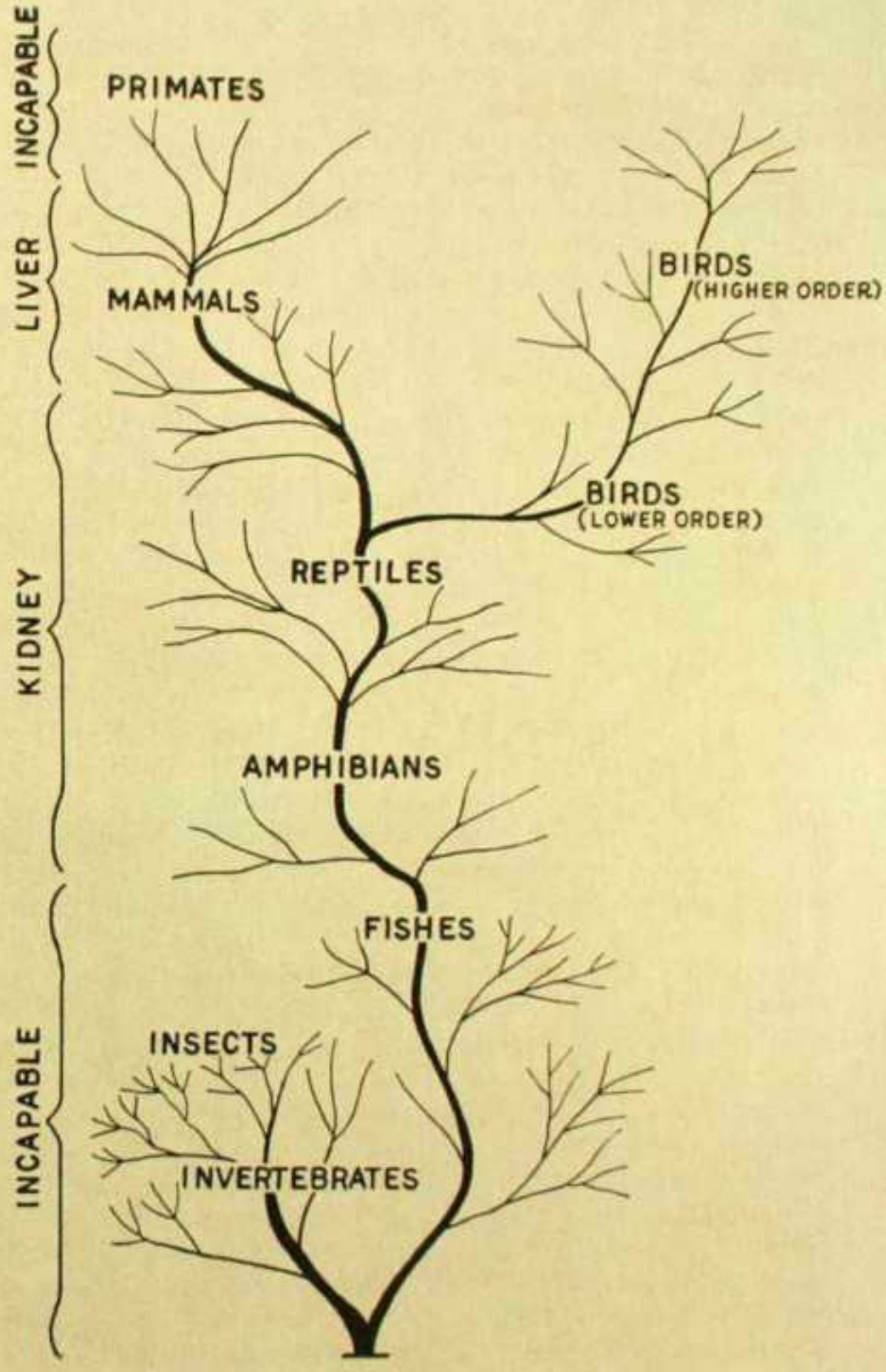
corbic acid was a must for overcoming the stressful conditions and the species with the biosynthetic ability survived competition.

Transition of the biosynthetic capacity from kidney to liver : The capacity to synthesize ascorbic acid by kidney of amphibians resided in the kidney of reptiles but transferred to the liver of mammals. The change of the site of synthesis from the kidney of reptiles took place when the vertebrates were evolving temperature regulatory mechanism and changed from cold blooded froms to the warm blooded species. The capacity of the kidney tissues had been altered to accomodate the necessities of life on dry land with increased physiological demands like regulation of urea, calcium, phosphate and other ions. It is possible that the relatively small kidneys became too crowded with these demands.

Though the activity of L-gulono oxidase (EC No. 1.1.3.8.) of kidney microsomal fractions from reptiles is comparable to that of the liver microsomal fractions from mammals, yet the net synthesis of ascorbic acid per kg body weight per day would be comparatively very much lower in reptiles. This is because the weight of kidney of reptiles is relatively small, only about 0.13-0.4 per cent of the body weight, whereas the liver of mammals comprises about 4-5 per cent of the body weight. The transition of the biosynthetic site from kidney to liver was apparently the evolutionary answer for the production of sufficient amount of ascorbic acid for maintenance of the mammals.

Loss of the biosynthetic ability : In the progress of evolution, the biosynthetic capacity is lost in the highest evolved species. The flying mammals are considered to be more near to the primates. The absence of biosynthetic capacity in this species supports the contention. The inability of the guinea pig may be explained by the consideration that the mutation in the guinea pig occurred independently.

The failure of the guinea pig, the flying mammals, monkey and man to synthesize ascorbic acid is due to a common defect, namely, the absence of the terminal enzyme L-gulono oxidase (8, 10, 18). This in turn may be attributed to the loss of the gene or the capacity of the gene responsible for synthesizing the enzyme. Whereas the biosynthetic capacity started in the amphibians which evolved roughly about 330-340 million years ago, the gene mutation leading to loss of the capacity took place in the common ancestor of man and other primates about 25 million years ago (19). The mutation leading to the loss of such an essential gene was, however, neutral (20) and not lethal. The mutants did not become extinct because the environment furnished the vitamin and the species continued to survive.


INCAPABLE
PRIMATES
LIVER
MAMMALS
BIRDS
(HIGHER ORDER)
BIRDS
(LOWER ORDER)
KIDNEY
REPTILES
AMPHIBIANS
FISHES
INCAPABLE
INSECTS
INVERTEBRATES

Need for Ascorbic acid

The desirable intake of ascorbic acid for maintenance of health is a subject of considerable controversy. The minimum protective dose that will prevent the development of scurvy in man is approximately 10 mg a day. However, available evidence suggests that the amount needed for maintenance of optimal health may be considerably greater than this. The Food and Nutrition Board of the National Research Council of the United States recommended a daily intake of 70-75 mg ascorbic acid for adults and 100 mg during pregnancy and lactation.

In contrast to the above recommendations, recently Pauling (21, 22) suggested that for optimum health the daily intake of ascorbic acid for an adult man should be 2.3 g which could be increased to 9-10 g in ailing conditions. This is about 30 to 130 times the dose recommended by the Food and Nutrition Board. According to Pauling, the fact that most mammals can synthesize ascorbic acid would indicate that for optimum health the need for the vitamin is much more than that can be provided by the usually available foodstuff. Pauling considered that though man has lost the biosynthetic ability, yet the need for the vitamin is similar to that of other mammals capable of synthesizing the vitamin, including the rat. The rate of production of ascorbic acid by rat is 28-56 mg per kg body weight per day (23, 24). Assuming that the same rate of production would be proper for a human being, Pauling (21) argued that a person weighing 70 kg should normally ingest between 1.8 g and 4.1 g per day. Pauling (22) further assumed that the common ancestor of man and other primates probably existed mainly on plant food and the average amount of ascorbic acid consumed daily by the ancestor was 2.3 g. To support this, he cited the example of Bourne (25) that the gorilla consumes about 4.5 g ascorbic acid per day.

However, the arguments of Pauling are questionable. The results presented elsewhere (14), would indicate that, the biosynthetic capacity of mammals decreased with progress of evolution. Since the biosynthetic capacity would apparently depend on the need, it may be well assumed that the need for ascorbic acid has decreased with the progress of evolution. Actually, studies with labelled ascorbic acid has shown that whereas the daily requirement for ascorbic acid per kg body weight of rat is 26 mg, that of the guinea pig is 9 mg and of human being only 1 mg (26). In his article on " Evolution and the need for ascorbic acid. Pauling (22) did not consider the possibility that the need might decrease with the progress of evolution. There was little relevance to compare the need of man with that of the rat which is about 26 times more.

Moreover, the daily consumption, of 4.5 g ascorbic acid by the gorilla and the hypothetical 2.3 g by the common ancestor of man and other primates could be just incidental through consumption of plant food and should not be considered as requirement for the vitamin. In experiments with rats and guinea pigs, we did not get any extra beneficial effect of large doses of ascorbic acid on growth and maintenance of the animals fed a fortified wheat diet with adequate intake of protein (27).

There are, of course, some clinical reports (26) that large doses of ascorbic acid are beneficial in a variety of stress conditions, namely, burns, injuries, surgery, trauma, cold, infections, rheumatic diseases, allergies, administration of drugs and endotoxins and in pregnancy. Recently, we have shown (28) that autooxidation of ascorbic acid in presence of histamine results in rupture of the imidazole ring leading to biological inactivation of histamine and we have indicated that the beneficial effect of large doses of ascorbic acid may be due to its detoxification of the excess histamine produced or released in the different stress conditions (29, 30). In the guinea pig, a maximum effect was obtained with a dose of 50 mg ascorbic acid per kg body weight per day, which is about 5 times the normal need of these animals.

The function of ascorbic acid to detoxicate histamine would probably justify the suggestion of Pauling for an increased intake of ascorbic acid for persons suffering from cold, allergy, infections and other infections and other stress conditions. However, the question still remains open. Are large doses really safe ? Ascorbic acid is usually considered to be nontoxic and harmless and Pauling (21) has taken the view that one can take any amount of ascorbic acid without the least danger and he did not hesitate to prescribe a dose 30-130 times that recommended by the Food and Nutrition Board. Perhaps, this would be harmself as long as the food consumed is nutritionally balanced with adequate intake of protein (27). On the other hand, in experiments with guinea pigs we recently observed that when the animals were fed a low protein high cereal diet, a daily iniake of 0.3 g or more ascorbic acid per kg body weight was toxic to the guinea pigs as revealed by inhibition of growth and early mortality (27). Or in other words, a dose of about 30 times the normal need was toxic to the guinea pigs fed a low protein high cereal diet. The majority of the world population is unable to afford the luxury of having animal protein in their diet and depends largely on grain crops. Therefore, if the results obtained with the guinea pigs are also applicable in human beings, then in geographic areas where the basal diet is consisted mainly of high cereals, daily intake of large doses of ascorbic acid may rather be harmful instead of being beneficial.

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NUTRITIONAL IMPROVEMENT AND NUTRITIONAL STANDARDS FOR FOODS

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Much information and knowledge have been gathered on the effects of malnutrition on human subject. By now it is also known how horrifying its effect is on a community. Its effects on children have been dealth with adequately (1). It inhibits physical growth, and also biochemical development which has been found to parallel the suppressed physical development to a considerable extent, neural growth and mentation and increases the incidence of infection. Owing to malnutrition nearly 60% of children do not become youths, their nerves and brains are not very active, their intelligence is retarded and there is proof that the latter cannot be made good even with adequate nutrition in the later years. It has also been known that the corrections of the damage done by malnutrition is usually impossible. McCance described the effects of malnutrition on growth, metabolism and final form (2) and Dobbing on the development of nervous system (3) and brought out the findings of damages done by malnutrition. Though the children, and for that matter the pregnant and nursing mothers, are the most vulnerable in this respect malnutrition does harm to the community by affecting the grown-ups also in different ways. It affects adversely the strength of population by premature deaths ; it decreases the physical productive performance in all forms of occupation ; it decreases the resistances to infection and hence increases absenteeism in working places. It affects, and sometimes inhibits, the development of intellect. Consideration of these adverse effects of malnutrition can be taken along with the accrued loss in national income and a sociologic calculation can be made on this adverse economy of malnutrition. The cost of treating a patient suffering from malnutrition is much more than that of providing him a nutritionally adequate diet ; it is known from an account that such a patient require 600 dollars for 90 days hospitalisation (in Guatemala) whereas annual expenditure for him to take preventive measure against malnutrition is not more than 7 to 10 dollars. When the difference in the two costs is staggering in the case of an individual victim, it is also possible to show that advance measure for provision of a nutritionally adequate diet on a national scale is less costly than the amount necessary for curative measures added with that of economic loss, even taking for granted that a fraction of population will be affected in absence of the advance national measure.

Planning for an adequate nutritional status through dietary means for a nation will certainly include the nutritional improvement of the common foods as one of the main measures among others. Of all the foods consumed by man none except milk (albeit its iron deficiency) can give them full nourishment. They are sustained by consuming a variety of foods selected from the plants and animals within their reach. Because of the technological development and attended sophistication nutritional loss occurs in foods (4). Flavour and palatability of food products may affect basic instincts and induce people to take foods which are less nutritious. For a number of reasons it may be difficult for a man to select a right combination of foods for him even with some amount of nutritional consciousness. Nutritional improvement of foods will be of advantage in this difficulty by producing highly nutritious foods at a low cost. These brief arguments will underline the rationale for nutritional improvement of foods.

Roy advocated nutritional improvement of foods much earlier (5). The need is still there for India ; now it is opportune that the project is possible because of increased consciousness and food technologists can accomplish the jobs whenever called for. Without going into details and further arguments it can be agreed that for a country like India nutritional improvement of foods should invariably be resorted to wherever possible. A few types food can be considered for nutritional improvement briefly.

Cereals, pulses and their products

In India at present enriched flour and atta are known and their nutritional specifications along with others have been laid down in the Indian Food Rules, 1955 (6). Two other products—multipurpose food and balahar—have been tailored with an eye to nutritional needs ; though their statutory standards have not been laid they are prepared under competent supervision. The bread is enriched with lysine, minerals and vitamins but only by a Government agency primarily.

But these existing activities can be extended by treading new fields which may include whole rice and wheat, breakfast cereals and macaroni products. Whole rice and wheat can be enriched with iron, thiamine, riboflavin and niacin (with a coating of colodian for rice) as premix or mastermix to be blended with rice and atta respectively at local mills or by the housewives. Riboflavin may be excluded, if exigent, in rice as it was found from experience in Formosa and Manila that rice with yellow spots were not liked by the housewives at the beginning who used to pick them out and discard them ; however, later it was reported that not only had the consumers' suspicion of the yellow spots been removed but that some said the yellow colour gave a sense of security (7).

In the U.S.A. a few nutritional standards—standard of identity for enriched bread, for enriched corn meals and grits, for enriched flour, for enriched farina, for enriched macaroni products, and for rice—exist to regulate the level and mode or enrichment.

Pulses and their powder (besan) can also be enriched as wheat and flour.

Enriching cereals and their products with proteins and amino-acids is another utility. Cereals being the main staple foods in the developing countries and the main source of protein in human diet, are the logical carriers for added protein. Worldwide cereals provide 40 million tons of protein as compared to 25 and 12 million tons from animal and legume sources. Isolates and concentrates from oilseeds, coconut, fish and single-cell proteins—and milk powder at least in principle if indigenous milk production can be increased—and amino acids, if economically synthesised and cheaply available, can be used for enriching powdered and prepared cereals. World protein resources have been reviewed and indicated in details (8).

Dairy products and Margarine

Milk is processed now-a-days in many dairies in India specially for urban milk supply. This provides an excellent medium for conveying a number of nutrients. Bottled fluid milk, toned milk and specially the low fat formulated and non-fat dry milk can be enriched with vitamins A and D, thiamine, riboflavin, niacin, ascorbic acid, iron and iodine according to local exigency.

Margarine, butter and ghee (except those used for cooking and baking) should be enriched with carotene or vitamin A. Vitamin D may not be used in these products being not of so much significance ; but fluid milk meant for infant use should have it. Enrichment of Vanaspati as practised in India and as demanded in the Indian Food Rules (6) can be stopped ; Roy after an extensive series of experiments on the stablity of carotene and vitamin A under different conditions of heating and cooking showed that a substantial loss of the nutrients occur though the use of antioxidants reduces the loss to some extent (9).

Sugar and sugar products

Candies and sweets are concentrated sources of carbohydrates. Being synthetic foods of crystalline sugar they contain little nutrients. Further, these are widely consumed by children. These should be enriched with major nutrients of carbohydrate metabolism *e.g.* thiamine, riboflavine and niacin. The Food and Nutrition Board of the National Research Council recommended an allowance of 6.6 mg of niacin or equivalent and 0.4 mg of thiamine per 1000 caloris. Taking twice this minimum requirement, sugar rich foods may contain 0.2 mg of thiamine and 3.6 mg of niacin per 100 gm

of sucrose in an attempt to make these foods metabolically self sufficient. Additionally these may contain cariostatic phosphates (0.5 to 1.0%) and iron.

However, the technological aspect of enriching sugar products should be considered. Usually these products receive drastic proeessing which may destroy significant amounts of the food nutrients and those added to sugar-containing foods as a part of the enrichment programme. Thiamine is rather unstable to heat specially under alkaline conditions and in the presence of oxidising agents. Niacin is somehwat more stable and phosphates are fairly stable. Each food will require special consideration to develop the best procedure for its enrichment, and enrichment of certain types of sweets may not be feasible unless change in the manufacturing process is admitted.

Fruit products

Almost all fruits provide ascorbic acid primarily and B vitamins to some extent ; some fruits are also sources of carotene. Hence logically enrichmen is envisaged to provide adequate supply of ascorbic acid. β-carotene and β-apo-8'-carotenal can be used to provide both colour and vitamin A value. Thiamine, riboflavin and niacin can be used specially to fruit products containing high concentration of sugar.

Special Dietrary Food products

These are designed to meet specific physiologic states and, in many situations, these may constitute a necessary and specified source of nutrient supply. Most special dietary foods are fed to relatively sensitive biological systems as illustrated by the rapidly growing human infants, the pregnant and lactating mothers, obese persons and the diabetic. These have to have the vitamins and minerals as dictated by the dietetic needs.

Nutritional improvement by decreasing nutrients

Another idea relevant in this context is 'denudation' or 'extraction' virtually opposite to what enrichment purports. Endeavour should be made to lessen the content of sugar, saturated fat and cholesteral contents of processed foods. Sugar even if supplemented with vitamins should be less than the present contents of foods like fruit products, dairy products and sweets and confectioneries. Saturated fat and cholesteral can be decreased by extraction of fat partially or wholely and subsequent supplementation with polyunsaturated fats, as in filled milk. In processed or formulated meat products stress can be laid on leaner meat or even on substitution of polyunsaturated fats for a part of the meat fat. This is an open idea and much thoughts and deliberations may precede action in this direction by the food industries, Government, public demand or regulatory standards.

Conclusion

The pernicious effects of malnutrition on a community is fairly well-known. Rationale for nutritional improvement on a national scale of foods has been briefly argued and established, it being a public health problem of major significance. Possible cases of nutritional improvement have been indicated. These can be put into effect in the form of nutritional standards which can be voluntary or persuasive as codes of practices or obligatory. As the practice (and hence probably the thought and awareness) is not adequate in India—albeit some references of obligatory nutritional standards in Indian food regulations (6) in respect of proteins and some vitamins—this article has been merely indicative and illustrative. If interest and activities in this regard increase, numerous details have to be worked out for selection of foods and determination of nutrients requiring improvement qualitatively and quantitatively depending on the national exigency.

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MYCOBACILLIN

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Since the discovery of penicillin, a very effective remedy against bacterial diseases, attention has naturally been drawn to the isolation of antifungal antibiotics to fight fungal infections both surface and systemic. By the time the work on mycobacillin was undertaken, a long list of antifungel antibiotics were already reported, but none of them obtained as single chemical enentities of determined structure. Mycobacillin[1] was the first antifungel polypeptide antibiotic to be isolated and its structure determined.

Isolation and Chemistry

In early fifties, an extensive research programme, in light of prevailing fungus infections in eastern part of India, was undertaken to screen out successful antifungal antibiotic producing organisms from Indian soils, vegetables and fruits which resulted in the isolation of one hundred seventy eight antifungal organisms. They were identified and found to belong to different genera, *Aspergillus, Penicillum, Alternaria, Steptromyces, Bacillus* and *Pseudomonas*[2,3]. A strain of *Bacillus subtilis* was tipped amongst these isolates as the most active producer of the antibiotic, later on designated as mycobacillin.

The antibiotic is a cylic peptide whose molecular weight, as calculated from its diffusion rate through a porous membrane is 1775 (which comes to 1527, when calculated from the molecular weights of constituent amino acids). The molecule which is essentially homogeneous contain 13 residues, of seven different amino acids whose sequence was determined by Sangers' method.

Mycobacillin was partially hydrolysed and the hydrolysate fractionated by two-dimensional paper chromatography for complete separation of this complex mixture of peptides. Altogether 15 peptides including seven dipeptides, six tripeptides and two tetra peptides were isolated. The N-terminal residue of each peptide was determined by both the deamination method and dinitro phenyl (DNP) method. Determination of the chemical structure of tri and tetra peptides without the application of stepwise degradation method or any method for determing C-terminal amino acids has been possible because some of the amino acids such as serine, alanine, leucine and proline are present in the proportion of one molecule each per molecule of mycobacillin. Since these amino acids occur only once in the molecule, the known structure of different dipep-

tides containing any one of these amino acids has been helpful in determining the possible arrangement of amino acids in tripeptides and tetrapeptides. This knowledge of chemical structure of di-, tri-, and tetra peptides has made it possible to fit together 15 peptide fragments from 13 amino acid residues of mycobacillin into an unique sequence (Fig. 1). In the absence of a free L-amino group in the molecule a cyclic structure has been deducted[4].

All the constituent glutamic acids of the molecule and 4 out of the 5 residues of aspartic acid are of D-configuration, the rest of the amino acids being of L-variety[3]. Thus the D-L-sequence of amino acids, in mycobacillin resolves itself to the determination of the D-L-sequence of aspartic acid only. The treatment of hydrolysates of aspartic acid containing peptides obtained from partial hydrolysis of mycobacillin with D-amino acid oxidase or L-glutamic acid decarboxylase (containing L-aspartic decarboxylase activity) revealed L-aspartic acid only in two peptides and D-aspartic acid in others. Hydrazinolysis shows that one such L-aspartic acid containing peptide is aspartyl-tyrosine and the other is (Asp., tyr) ser. Majumdar and Bose had shown by reaction with florodinitrobenzene that the peptides with similar Rf values both had N-terminal aspartic acid. Hence the single L-aspartic acid in mycobacillin must occupy oposition 5 in the complete structure of the molecule shown in Fig. 1[4].

It is now generally accepted that the predominant chemical bond in proteins is the α-peptide linkage. Studies of naturally occurring small peptides and peptide antibiotics indicate that some of these have peptide linkages involving the side-chains of amino acids. Mycobacillin contain seven residues of two dicarboxylic amino acids *viz.* aspartic and glutamic acids in the molecule. Potentiometric titration and chemical assay of free carboxyl groups indicate that the antibiotic lacks amino group but contains two free α-carboxyl groups indicating the presence of two side-chain peptide linkages, which may be either β-or γ or both. Hypobromite oxidation of the intact molecule also indicates the presence of at least one γ-peptide linkage. Further confirmation of γ-or other types of peptide linkage or quantitation of the number of γ-peptide linkages of the molecule were done by hydrazinolysis and also by reduction of esterified mycobacillin followed by hydrolysis and identification. All those methods conclusively proved that all aspartic acid residues are L-linked and glutamic acids γ-linked in mycobacillin. Mycobacillin does not react with hydroxylamine to give any hydroxamate indicating the absence of anhydride, lactone and ester linkages in the molecule. This is also confirmed by IR spectroscopy and titration of the molecule. The structure of the molecule so far elucidated is shown in Fig. 1.

Biosynthesis

Studies on biosynthesis of mycobacillin was undertaken in 1961 when a well-knit hypothesis to account for the biosynthesis of protein has already been formulated and that of small peptides like glutathione by non-template mechanism was just been confirmed[7]. But few informations were available at that time regarding the biosynthesis of antibiotic peptides except for the fact that RNA template is not involved in biosynthesis of gramicidin S[8]. In this context the biosynthesis of a peptide of the size of mycobacillin which is about one fourth the molecular weight of insulin possess an interesting query. Chloramphenicol, a well-established inhibitor of protein synthesis, inhibits both growth and mycobacillin formation and so its effect could not be interpreted. But when nucleic acid base antagonists were added at the 66th hour of growth in stationary culture, growth, RNA and DNA synthesis stop, whereas mycobacillin production continues as usual indicating that RNA of high molecular weight is not involved in the formation of antibiotic. A change in the concentration of labelled aspartic acid in metabolic pool leads to a corresponding change in the specific activity of aspartic acid in the different peptide fragments of mycobacillin molecule suggesting that mycobacillin synthesis probably occurs by linear addition of amino acids[9].

A streptomycin-dependent variant was isolated from the antibiotic producer strain by single-step mutation process. Streptomycin inhibited the growth of the mutant in concentrations exceeding an optional level. It is also observed that when this mutant had been grown in presence of streptomycin but subsequently deprived of it, mycobacillin production remains quite normal, but protein synthesis is seriously impaired. This non-interference of streptomycin deprivation, may therefore, be taken as a further evidence in support of the previous ocnclusion that ribosome does not take part in mycobacillin biosynthesis[10].

The constituent D-amino acids of mycobacillin do not inhibit its synthesis[11] in the whole cell fermetation. Studies with different non-producer mutants obtained by ultraviolet irradiation of the parent strain showed that none of the possible combinations between two mutant strains produces mycobacillin[12].

The cell-pool of the producer organism was found to contain four nucleotide peptides. Uracil was present in all these nuclectide peptides whose amino acid composition resembles that of mycobacillin suggesting a probable relation between the two[13].

An effective cell-free system, prepared by protoplast lysis of the producer strain was developed. The incorporation of labelled amino acids into mycobacillin by the system is energy dependent and insensitive to RNase action[14].

In the case of gramicidin S and a non-antibiotic cylic penta peptide malformin, has the enzyme system responsible for their synthesis been shown to be localised in the soluble supernatant of the producer organism. It is observed that enzyme systems participating in mycobacillin synthesis are localised in the soluole supernatant and ribosomes have no effect. Inhibitors of protein synthesis such as chloramphenicol, streptomycin and puromycin have no effect on mycobacillin synthesis in cell-free system. K^+, NH_4^+ and non-mycobacillin amino acids appear to be neither stimulatory nor inhibitory, whereas mercaptoethanol stimulates synthesis[13].

Enzymatic synthesis of peptide bond is a energy consuming process and requires participation of ATP. In general, the ribosomal peptide bond synthesis occurs through the unique participation of energy rich amino-acyl adenylate formed from amino acids and ATP. On the other hand no such generalization could be made regarding the mode of energy consumption in the non-ribosomal synthesis of small peptides and peptide antibiotics. In the biosynthesis of small peptide like glutathione, ophthalmic acid and cell wall peptide, amino acids are added in the peptide chain with concomitant breakdown of ATP into ADP and P_i, whereas the biosynthesis of peptide antibiotics like gramicidin S, tyrocidine requires participation of aminoacyl adenylate not only of L-amino acids but also of D-amino acids as indicated by L- and D-amino acid dependent ATP—PP_i exchange[14]. But no such D-amino acid dependent ATP—PP_i exchange[15] could be identified in the cell-free system of the organisms producing polymyxin, actinomycin or circulin, though they require ATP for peptide bond formation.

The enzyme system synthesising mycobacillin fails to catalyse any ATP—PP_i exchange in presence of D-amino acid of mycobacillin although D-glutamic acid is a better precursor than its L-isomer[16]. All mycobacillin L-amino acids are however activated as usual. Now in course of our studies on different modes of ATP breakdown for peptide bond formation, it is observed that the enzyme system synthesing mycobacillin catalyse L-proline dependent ATP—P_i exchange and that this exchange is significantly stimulated by other mycobacillin amino acids, if they are added sequentially from L-proline[18]. This stimulation is, however, inhibited by the deprivation from the amino acid mixture of D-aspartic acid, the amino acid next to L-proline.

In the biosynthesis of cyclic peptides, it is very pertinent to ask which of the amino acids of mycobacillin is the initiating point in the synthesis. Isolation of a series of peptides from the enzyme system incubated with suitable mixture of amino acids and elucidation of their structures have made it possible to suggest D-phenylalamine as the point of initiation and subsequent growth

leading to the formation of cyclic peptides like gramicidine S or tyrocidine[21]. Now in the light of these observations on L-proline dependent ATP—P_i exchange and its sequential stimulation, we have studied the condition of inhibition of stimulations, caused by mycobacillin amino acids on ATP—P1 exchange, initiated by L-proline and also the fate of mycobacillin synthesis by usuing sequential amino acid deprivation technique starting from L-proline.

In a cell-free system[13] from *Bacillus subtilis* B_s, ATP—P_i exchange was catalysed by L-proline at a pH otpimum of 7.2. Further stimulation by component amino acids of mycobacillin was inhibited by deprivation from the synthesising system of even a single a mino acid occurring at any point of the cyclic peptide. This inhibition however decreased with distance in the molecule of the given amino acid from L-proline. Peptides containing respectively two, three, four, five and six amino acids were isolated from the mycobacillin synthesising system by amino acid deprivation technique. The amino acid composition of these peptides, their C- and N-terminal amino acid residues and also stereo-configuration[22] were the same as those of peptides that would be obtained if mycobacillin synthesis occurred starting from L-proline and was interrupted at various points along the polypeptide chain. How the sequence of the polypeptide in absence of ribosomal synthesis is determined remains still a mystery.

Significance of antibiotic synthesis

It is very relevant to ask the significance of antibiotic on the life process of a producer antagonist. How does an antibiotic affect a producer ? Reports on the effect of antibiotic on producer organisms are very few. Growth inhibition at concentrations much higher than those required for sensitive organisms has been reported from various laboratories although the protoplasts of producer and sensitive organisms respond equally to the action of some antibiotics. Some times low yielding strains are more sensitive than high yielding ones as in the case of *S. griseus* towards streptomycin[23].

It is observed that growth of producer *B. subtilis* B_s is not supported in a synthetic medium when mycobacillin is incorporated as a nitrogen source. The antibiotic induces a lag in the growth of a producer or a nonproducer strain of *B. subtilis*. The producer organism also responds, to some extent, to mycobacillin with respect to its sensitive reactions like release of UV-absorbing materials, loss of gram positive character, agglutination, etc. Cells of *B. subtilis* B_s in early log phase of growth are more sensitive to mycobacillin than those in late log phase or post log phase of growth while spores are completely insensitive to it. Though whole cells of the producer or a nonproducer strain of *B. subtilis* show little release of UV-absorbing materials on exposure to myco-

bacillin, protoplasts of both the strains do so to a considerable extent. The permeability of cells of *B. subtilis* is altered to the effect that retentivity of dyes like methylene blue is reduced. Thus the difference regarding the response of the producer and the sensitive organisms towards mycobacillin appears to be quantitative rather than qualitative[23].

Significance was then sought for in terms of basic cellular metabolism. Most of the bacteria that produce peptide antibiotics are aerobic spore-former. The interrelation between the two processes was suggested by different laboratories. Bernlohr and Novelli[24] reported that the polypeptide antibiotic bacitracin is incorporated intact into the spore coat. Conflicting reports are however available. Hence it was considered worth while to reappraise the whole issue using *B. subtilis* which produces mycobacillin, an antibiotic peptide of almost the same size as bacitracin.

Both mycobacillin production and sporulation are initiated in the post log phase of growth of *B. sublitis* B_1, reaching their maximum by about the same time. Mycobacilin is released during endotropic sporulation of vegetative cells, though not during germination of spore in complex growth medium11. Similar observations were also obtained by Bernlohr and Novelli[25] Antisporogenic chemicals like glucose (in excess), diethylmalonate, acriflavin, fluoroceaic ac[1] ,sodium bisulfite, β-phenethyl alcohol, α-picolinic acid and m-tyrosine inhibit mycobacillin production.[26] Bernlohr Novelli[24], and Paulus[27] also observed the inhibition of antibiotic synthesis by antisporogenic chemicals. By acriflavin and actinomycin D treatment, two types of mutants, oligosporous and asporogenous, were obtained. Mycobacillin production is affected adversely in oligosporous mutants whereas asporogenous mutants do not produce the antibioiic at all. Schaeffer[28] also isolated several Sp^-Ab^- mutants. Sporogenesis and antibiotic synthesis whose close association appears to be indicated by the inhibition of both the processes by common inhibitors and also by lack of antibiotic production by asprogenous mutants are however completely dissociated in Sp^-Ab^+[28],[29], and in Sp^+My^- mutant in the authors' laboratory[30].

During sporulation specific cell wall lytic enzymes[31] developed which cause considerable amount of lysis of cell walls and release of constituent peptides[32]. Now the amino acid composition of cell wall or spore count of *B. subtilis* B_1 differs both qualitatively as well as quantitatively from that fo mycobacillin. Therefore mycobacillin can not be assumed to be a structural component of cell wall or spore coat of the producer *B. subtilis* B_1[33]. The conclusion is consistent with that of *Snoke*[34] and *Brenneer*[35] with regard to bacitracin— and polymyxine B-producing *B. lichenitormis* and *B. polymyxa* respectively.

Thus the close association as also disociation between sporulation and antibiotic synthesis has yet to be assessed both genetically and biochemically.

Mode of action

The genus *Bacillus* elaborates a number of polypeptide antibiotics. These are generally antibacterial whereas mycobacillin, a polypeptide antibiotic also elaborated by the same genus, is exclusively antifungal. A good number of antifungal antibiotics are known but only a few of them except those of the polyene type have been investigated with regard to the mode of action. Different polyene antibiotics seem to act by altering cellular permeability[36]. Polymyxin a cyclic peptide not antifungal, but antibacterial is supposed to act on the osmotic barrier of sensitive cells[37].

Mycobacillin is a broad spectrum antibiotic being active against skin pathogens, plant pathogens and also saprophytic fungi. Regarding its action on a sensitive strain of *Candida albicans*[38] it has been observed that it enhances lag period[6] but does not affect energy yielding processes like respiration and glycolysis. Though mycobacillin slightly affects the oxidation of its constituent amino acids, protein synthesis as measured in terms of incorporation of labelled leucine, serine or glycine into TCA precipitable fraction, remains unaltered in its presence. None of the constituent amino acids of mycobacillin can antagonize the growth inhibiting property of the antibiotic. It brings about agglutination of cells of a sensitive strain *Candida albicans*, changes its Gram-character and causes release of UV-absorbing materials from the cells.

Now detailed kinetic studies on mycobacillin sensitive reactions indicate that visible agglutination lags behind fall in viability which is also not quantitatively related to release. Thus none of the above mycobacillin-sensitive reactions alone can be considered as responsible for the antifungal action of the compound.

It is observed that sterols *e.g.* cholesterol, ergosterol, ergocalciferol and also lecithin can antagonise the growth inihibitory property as well as the leakage action of mycobacillin.[39] The action of polyene antibiotic has also been reported to be antagonised by sterols and lipids. Lipid like compounds were isolated from a mycobacillin sensitive organism *A. niger* and fractionated into neutral and phospholipid components which were found to contain mainly cholesterol and lecithin respectively. Both these lipid fractions isolated from the same very sensitive organism antagonise the growth inhibiting property of mycobacillin and may therefore be considered as the binding site for the compound. The antibiotic does not interfere with their biosynthesis. Sterols and lipid exert their antagonistic action, if added at O hr, subsequent addition fails to antagonise mycobacillin action. Electrophoretic, chromatographic

and spectrophotometric behaviour of mycobacillin, cholesterol and lipid either alone or in combination of their respective antagonising concentrations, indicates that antagonism might result from chemical interaction between the antibiotic and antagonist. Similar hypothesis has been advanced to account for the antifungal action of polyenes[45] which are chemically different from peptide. Studies with cholesterol derivatives and lecithin components indicate that 3-hydroxyl group must be free for cholesterol to act as an antagonist and that unsaturated oleic acid is the reactive component of lecithin[41]. There still remain the questions regarding the chemical nature of materials released by mycobacillin, nature of the reactive groups involved in lipid antibiotic antaganisation reaction and finally the subsequent effect of this very interaction on the dynamic structure and function of membrane.

Evaluation of mycobacillin or its derivatives as an antifungal drug

Polypeptide antibiotics are generally toxic. Mycobacillin is greatly inactivated in presence of serum which limits its possible use as a drug. Reduction in hemolytic action of gramicidin has been variously reported. Acetylation lowers antifungal activity of mycobacillin, the inhibitory concentration (mg/ml) for the di-and tri-acetyl derivatives being 35-40 and 40-48 respectively as against 15-20 for mycobacillin ; but acetylation gives complete protection against serum inactivation of the antibiotic whose inhibitory concentration is increased tenfold in its presence[42]. Esterification of mycobacillin with different alcohols decreases its antifungal activity as otherwise observed for esterified subtilin. However, esterification protects it partially from inactivation by serum. An ideal derivative has yet to be looked for.

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